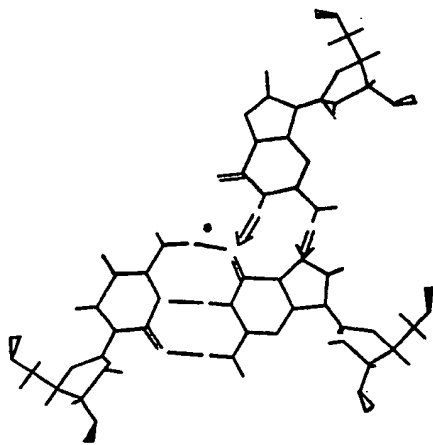


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(54) Title: TRIPLE STRANDED NUCLEIC ACID AND METHODS OF USE  (57) Abstract <p>A method for making synthetic oligonucleotides which bind to target sequences in a duplex DNA forming colinear triplexes by binding to the major groove. The method includes scanning genomic duplex DNA and identifying nucleotide target sequences of greater than about 20 nucleotides having either about at least 65% purine bases or about at least 65% pyrimidine bases; and synthesizing synthetic oligonucleotides complementary to identified target sequences. The synthetic oligonucleotides have a G when the complementary location in the DNA duplex has a GC base pair and have a T when the complementary location in the DNA duplex has an AT base pair. The synthetic oligonucleotides are oriented 5' to 3' and bind parallel or 3' to 5' and bind anti-parallel to the about at least 65% purine strand. Also described are synthetic oligonucleotides made by the above methods. The oligonucleotides can be altered by modifying and/or changing the bases, adding linkers and modifying groups to the 5' and/or 3' termini, and changing the backbone. These synthetic oligonucleotides bind to duplex DNA to form triplexes. This process alters the functioning of the genes which are bound. This process can be used to inhibit cell growth, alter protein ratios, treat diseases including cancer and permanently alter the DNA.</p>		

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Triple Stranded Nucleic Acid and Methods of Use

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This application is a Continuation-in-Part of Applicants Co-pending U.S. Application Serial No. 287,359, filed December 20, 1988.

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This invention was supported in part through a grant or award from the National Institute of Health.

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FIELD OF INVENTION

The present invention relates generally to a method for making synthetic oligonucleotides which bind to the major groove of a duplex DNA to form a colinear triplex. It also relates to synthetic oligonucleotides which bind to the purine strand of a DNA duplex. It

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1 further relates to a method of regulating and inhibiting
cellular growth by administering a synthetic
oligonucleotide which is capable of binding to a DNA
duplex to form a colinear triplex.

5

BACKGROUND ON THE INVENTION

It has been known for some time that the
polynucleotide polydT will bind to the polydA-polydT
10 duplex to form a colinear triplex (Arnott, S & Selsing E.
(1974) J. Molec. Biol. 88, 509). The structure of that
triplex has been deduced from X-ray fiber diffraction
analysis and has been determined to be a colinear triplex
(Arnott, S & Selsing E. (1974) J. Molec. Biol. 88, 509).
15 The polydT strand is bound in the parallel orientation to
the polydA strand of the underlying duplex. The
polydT-polydA-polydT triplex is stabilized by T-A
Hoogsteen base pairing between A in the duplex and the
third strand of polydT. That interaction necessarily
20 places the third strand, called a ligand, within the major
groove of the underlying duplex. The binding site in the
major groove is also referred to as the target sequence.

Similarly, it has been shown that polydG will
25 bind by triplex formation to the duplex polydG-polydC,
presumably by G-G pairing in the major helix groove of the
underlying duplex, (Riley M., Mailing B. & Chamberlin M.
(1966) J. Molec. Biol. 20, 359). This pattern of
association is likely to be similar to the pattern of
30 G-G-C triplet formation seen in tRNA crystals (Cantor C. &
Schimmel P., (1980) Biophysical Chemistry vol I, p.
192-195).

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1 Triplexes of the form polydA-polydA-polydT and
polydC-polydG-polydC have also been detected (Broitman S.,
Im D.D. & Fresco J.R. (1987) Proc. Nat. Acad. Sci USA 84,
5120 and Lee J.S., Johnson D.A. & Morgan A.R. (1979) Nucl.
5 Acids Res. 6, 3073). Further the mixed triplex
polydCT-polydGA-polydCT has also been observed. (Parseuth
D. et al. (1988) Proc. Nat. Acad. Sci. USA 85, 1849 and
Moser H.E. & Dervan P.B. (1987) Science 238, 645). These
complexes, however, have proven to be weak or to occur
10 only at acid pH.

 Parallel deoxyribo oligonucleotide isomers which
bind in the parallel orientation have been synthesized
(Moser H.E. & Dervan P.E. (1987) Science 238, 645-650 and
15 Rajagopol P. & Feigon J. (1989) Nature 339, 637-640). In
examples where the binding site was symmetric and could
have formed either the parallel or antiparallel triplex
(oligodT binding to an oligodA-oligodT duplex target), the
resulting triplex formed in the parallel orientation
20 (Moser H.E. & Dervan P.E. (1987) Science 238, 645-650 and
Praseuth D. et al. (1988) PNAS 85, 1349-1353), as had been
deduced from x-ray diffraction analysis of the
polydT-polydA-polydT triplex.

25 Studies employing oligonucleotides comprising the
unnatural alpha anomer of the nucleotide subunit, have
shown that an antiparallel triplex can form (Praseuth D.
et al. (1988) PNAS 85, 1349-1353). However, since the
alpha deoxyribonucleotide units of DNA are inherently
30 reversed with respect to the natural beta subunits, an
antiparallel triplex formed by alpha oligonucleotides
necessarily follows from the observation of parallel
triplex formation by the natural beta oligonucleotides.
For example, alpha deoxyribo oligonucleotides form

1 parallel rather than antiparallel Watson-Crick helices
with a complementary strand of the beta DNA isomer.

5 It has been demonstrated that a DNA
oligonucleotide could bind by triplex formation to a
duplex DNA target in a gene control region; thereby
repressing transcription initiation (Cooney M. et. al.
(1988) Science 241, 456). This was an important
10 observation since the duplex DNA target was not a simple
repeating sequence.

The present invention provides a new method for
designing synthetic oligonucleotides which will bind
tightly and specifically to any duplex DNA target. When
15 the target serves as a regulatory protein the method can
be used to design synthetic oligonucleotides which can be
used as a class of drug molecules to selectively
manipulate the expression of individual genes.

20 SUMMARY OF THE INVENTION

The object of the present invention is a method
for designing synthetic oligonucleotides which bind to
duplex DNA.

25 A further object of the present invention is a
method for making synthetic oligonucleotides which form
triplexes with DNA.

30 An additional object to the present invention is
a synthetic oligonucleotide which forms a colinear triplex
with a target sequence in a duplex DNA.

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1 Another object to the present invention is a
provision of a synthetic oligonucleotide which inhibits
the growth of cells.

5 A further object of the present invention is a
provision of a synthetic oligonucleotide which inhibits
the growth of a pathogen.

10 An additional object of the present invention is
a method for altering the structural protein content of
epidermal tissue for the treatment of aging and blood
clotting.

15 A further object of the present invention is a
method of inhibiting gene expression by permanently
altering the DNA sequence.

20 Thus, in accomplishing the foregoing objects,
there is provided in accordance with one aspect of the
present invention a method for making a synthetic
oligonucleotide which binds to a target sequence in duplex
DNA forming a colinear-triplex by binding to the major
groove, said method comprising the steps of: scanning
genomic duplex DNA and identifying nucleotide target
25 sequences of greater than about 20 nucleotides having
either about at least 65% purine bases or about at least
65% pyrimidine bases; and synthesizing said synthetic
oligonucleotide complementary to said identified target
sequence, said synthetic oligonucleotide having a G when
30 the complementary location in the DNA duplex has a GC base
pair, having a T when the complementary location of the
DNA duplex has an AT base pair. In specific embodiments
the synthetic oligonucleotide can be selected from the
group consisting of an oligonucleotide oriented 5' to 3'

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1 and binding parallel to the about at least 65% purine
strand, or an oligonucleotide oriented 3' to 5' and
binding anti-parallel to the about at least 65% purine
strand.

5 A further aspect of the present invention is the
synthetic oligonucleotide for forming a colinear triplex
with a target sequence in a duplex DNA when said target
sequence is either about at least 65% purine bases or
10 about at least 65% pyrimidine basis, comprising, a
nucleotide sequence of at least about 20 nucleotides; said
nucleotide sequence including G and T, wherein G is used
when the complementary location and duplex DNA has a GC
base pair and T is used when the complementary location in
15 the duplex DNA is an AT base pair; and said sequence
selected from the group consisting of an oligonucleotide
oriented 5' to 3' and binding parallel to the about at
least 65% purine strand of the duplex DNA target sequence,
and an oligonucleotide oriented 3' to 5' and binding
20 anti-parallel to the about at least 65% purine strand in
the duplex DNA target sequence.

In the preferred embodiments the synthetic
oligonucleotide can have at least one T replaced by X, I,
25 and halogenated derivatives of X and I. Furthermore, at
least one G can be replaced with halogenated derivatives
of G.

Additional embodiments include substitutions on
30 the synthetic oligonucleotide. For example, the base can
be substituted at the 2' furanose position with a
non-charged bulky group and the backbone of the synthetic
oligonucleotide can be a phosphodiester analogue which is
not readily hydrolyzed by cellular nucleases. In

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1 addition, a linker can be affixed at the 3' and/or 5'
terminus of the synthetic oligonucleotide. This linker
provides a method for attaching modifying groups to the
oligonucleotide. The modifying groups can be
5 intercalators, groove-binding molecules, cationic amines
and cationic polypeptides.

Another aspect of the present invention is a
method of inhibiting the growth of cells comprising the
10 step of administering synthetic oligonucleotides in
sufficient quantity for cellular uptake and binding to the
target sequence, wherein said target sequence is
positioned within the DNA domain adjacent to the RNA
transcription origin. This procedure can be used to
15 inhibit the growth of cancer cells and pathogens. In one
preferred embodiment this procedure is used to inhibit
HIV-I virus by binding a synthetic oligonucleotide to the
viral LTR region.

20 Another aspect of the present invention is a
method of altering the relative proportions of the
structural protein content of epidermal tissue by
administering a synthetic oligonucleotide in sufficient
quantity for cellular uptake and binding to target
25 sequences for collagen genes.

Other and further objects, features and
advantages will be apparent from the following description
of the presently preferred embodiments of the invention
30 given for the purpose of disclosure when taken in
conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

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Fig. 1A shows the surface morphology of a colinear triplex. It is a computer generated rendering of the structure of a duplex DNA target site and presents in both the canonical B and A helix form. Upon binding of an oligonucleotide ligand, the target undergoes a transition from the B to the A form, which creates an increase in the depth of the major helix groove (M). In a colinear triplex, the oligonucleotide wraps about the A form helix target, occupying the major groove. The groove binding has been emphasized by presenting the bound oligonucleotide as a ribbon-like abstraction.

Fig. 1B shows the strand orientation in a colinear triplex. The oligonucleotide ligand binds to the duplex target, in the parallel orientation relative to the orienting (more purine rich) strand.

Fig 2 shows the pattern of oligonucleotide hydrogen bonding with the duplex target: G to GC sites, T to AT sites. 2A is a computer simulated rendering of the preferred pattern of hydrogen bonding between G in the ligand and G in the GC base pair at the corresponding site within the orienting strand of the duplex target. 2B is an equivalent simulation of T binding to the A of an AT base pair at its corresponding site within the orienting strand of the duplex target. The T-AT association is identical to classical "Hoogsteen base pairing", whereas the G-GC association is essentially the guanine counterpart thereof and involves N3 to O6 bonding. Solid wedges define the site at which such a crosssection through a triplex is affixed to the corresponding crosssection above it. Open wedges define the site at which such a

1 crossection through a triplex is affixed to the
corresponding crossection below. As seen, the
connectivity defined by the two bonding schemes is nearly
identical. It is also important to recognize that the
5 favored pattern of bond formation between G and GC or T
and AT (arrows) cannot be mimicked by any other pattern of
base-base association at neutral pH (C can mimic G in acid
conditions).

10 2C and 2D are corresponding bonding patterns
which result when the G of a GC base pair or A of an AT
pair occurs across from the orienting strand of the target
duplex. In that instance, the rules of oligonucleotide
sequence selectivity are the same (i.e., G at a GC pair, T
15 at an AT pair) however, G bonding occurs N3 to N9 and T
bonds in the "reverse Hoogsteen" way, thereby both retain
the overall parallel orientation of the bound ligand and
the orienting strand of the target.

20 Fig. 3 shows one method of improving the pattern
of oligonucleotide hydrogen bonding with the duplex
target: xanthine binding to AT sites. The computer
generated simulation in Fig. 3 is as in Fig. 2, except
that the effect of substituting xanthine (X) for T is
25 presented. As seen, in both the "Hoogsteen" binding (3A
and 3B) and "Reverse Hoogsteen" (3C and 3D) mode of
binding, X and T bind equivalently to an underlying AT
base pair. The major difference between the two is that X
is nearly identical to the G residues which might flank it
30 in an oligonucleotide ligand, with respect to base size
and shape and with respect to the orientation of its
phosphodiester component within the oligonucleotide
binding site. Modeling predicts that such enhancement of
oligonucleotide continuity will enhance the binding
35 affinity and site specificity of all oligonucleotides in
which T is replaced by X.

1 Fig. 4 displays the family of altered
phosphodiester linkages compatible with colinear triplex
formation. Some of the homologues of the phosphate within
the backbone of an oligonucleotide are presented. In each
5 instance, examples are cited which can be prepared by a
simple modification of the standard computer assisted,
solid phase methods. Examples A-C are thiophosphate
linkage, E is methylphosphonate, F is phosphoramidite and
G is phosphotriester.

10 Fig. 5 shows formation of hybrid oligonucleotides
by means of coupling through a 5' amine linkage. In this
instance, a hexylamine linkage is described. This linkage
can be affixed as the last residue of an oligonucleotide
15 by employing the same phosphoramidite chemistry used to
polymerize the DNA bases. After purification of the
linker-modified oligonucleotide, groups which selectively
react with a primary alkyl amine can be added. These
groups include the isothiocyanate derivative of eosin
20 (EITC) or 9 amino acridine (AIT), or any number of other
small molecules. Essentially identical chemistry is
available for affixing a thiol group to the 5' terminus.

25 Fig. 6 shows dose dependent inhibition of HIV-1
mRNA by Oligonucleotide mediated DNA triplexes.
U937/HIV-1 cells (ATCC CRL 1593, American Type Culture
Collection, Rockville, MD), infected with the HTLV-IIIB
prototype strain of HIV-1 and cultured under conditions
where >90% of the cells remained viable and contained
HIV-1 mRNA as shown by in situ hybridization with the
30 ³⁵S-labeled probe for the LTR of HIV-1, (NEP 200,
DuPont, Wilmington, DE)] were incubated with each
oligonucleotide at 0, 2, 6, 10, and 20 uM concentrations.
Oligonucleotide was added to the culture supernatants at

1 the initiation of incubation and again after 2 hours.
Cells were harvested after 4 hours incubation, and washed
with PBS before harvest of total cellular RNA using RNazol
(Cinna/Biotech Laboratories International, Inc.,
5 Friendswood, TX). Serial 2-fold dilutions were made from
each RNA preparation (starting at 2.5 ug RNA) and equal
amounts were applied to duplicate nylon membranes using a
slot blot apparatus (Biorad). One blot was probed with
the radiolabeled EcoRI-HhaI env fragment from the HIV-1
10 containing plasmid pARV-7/2, while the other was probed
with radiolabeled cDNA for β -actin. The resulting
autoradiographs were then analyzed by densitometry. The
density units expressed on the ordinate express the ratio
of (env-probe density) / (actin-probe density). Δ
15 represent HIV29par, \square represent HIV31 anti, and \square
represent random HIV29 isomer.

Fig. 7 shows the persistence of the effect of
oligonucleotides on HIV infected H9 T cells. HIV-1
20 infected U937 cells were cultured for 12 to 72 hrs. after
the last addition of HIV31anti. The oligonucleotide was
added at the initiation of the culture and at 2 hrs.
thereafter to maintain a final concentration of 10 μ M.
Cells were harvested at the indicated time points
25 thereafter. Total cellular RNA was harvested and applied
to duplicate nylon membranes in serial dilution with a
slot blot apparatus. One replicate was probed with the
HIV-1 env cDNA and the other with the cDNA for β -actin.
The density units (ordinate) are expressed as the ratio of
30 env to β -actin densitometry readings. \square represent
HIV31 anti and O represent controls.

1 Fig. 8 shows inhibition of viral mRNA by HIV29par
in infected H9 cells. The densitometric analysis shows a
decrease in specific viral message. H9 cells, infected
with HTLV IIIB, were treated with oligomer (5 μ M) every
5 two hours. At four and twelve hours the cells were
harvested, washed with PBS, and the total cellular RNA was
extracted. The hatched bars represent oligomer treatment
and unhatched bars represent controls.

10 The drawings are not necessarily to scale.
Certain features of the invention may be exaggerated in
scale or shown in schematic form in the interest of
clarity and conciseness.

15 DETAILED DESCRIPTION

It is readily apparent to one skilled in the art
that various substitutions and modifications may be made
to the invention disclosed herein without departing from
20 the scope and spirit of the invention.

25 The term "synthetic oligonucleotides as used
herein is defined as a molecule comprised of two or more
deoxyribonucleotides or ribonucleotides, preferably more
than ten. Its exact size will depend on many factors,
including its specificity and binding affinity.

30 When referring to bases herein the term includes
both deoxyribonucleic acids and ribonucleic acids. The
following abbreviations are used: "A" refers to adenine as
well as its deoxyribose derivatives, "T" refers to thymine
as well as its deoxyribose derivative, "G" refers to
guanine as well as its deoxyribose derivative, "C" refers
to cytosine as well as its deoxyribose derivative, "X"

1 refers to xanthine as well as its deoxyribose derivative
and "I" refers to inosine.

5 The "major groove" refers to one of the grooves
along the outer surface of the DNA helix which is formed
because the sugar-phosphate backbone extends further from
the axis than the bases do. The major groove is important
for binding of regulator molecules to specific DNA
sequences.

10 A set of procedures have been established to
design DNA or RNA oligonucleotides which bind specifically
to a DNA target by colinear triplex formation. One
embodiment of the present invention is a method for making
15 a synthetic oligonucleotide which binds to a target
sequence in duplex DNA forming a colinear triplex by
binding to the major groove, said method comprising the
steps of: scanning genomic duplex DNA and identifying
nucleotide target sequences of greater than
20 20 nucleotides, said target sequences having either about
at least 65% purine bases or about at least 65% pyrimidine
bases; and synthesizing said synthetic oligonucleotide
complementary to said identified target sequence, said
synthetic oligonucleotide having a G when the
25 complementary location in the DNA duplex has a GC base
pair, having a T when the complementary location in the
DNA duplex has an AT base pair. In specific embodiments
the synthetic oligonucleotide is selected from the group
consisting of an oligonucleotide oriented 3' to 5' and
30 binding anti-parallel to the about at least 65% purine
strand and an oligonucleotide oriented 5' to 3' and
binding parallel to the about at least 65% purine strand.
The resulting oligonucleotide can be synthesized in gram
quantities by the standard methods of solid phase
35 oligonucleotide synthesis.

1 The site-specific oligonucleotide procedure is
divided into three parts:

- I. Oligonucleotide base sequence design.
- II. Analysis of the duplex target
- 5 III. Secondary chemical modification of the
oligonucleotide.

I. Oligonucleotide base sequence design.

10 After identifying a DNA target with an
interesting biological function, an oligonucleotide length
must be chosen. There is a one to one correspondence
between oligonucleotide length and target length. For
example, a 27 base long oligonucleotide is required to
15 bind to a 27 base pair long duplex DNA target. Under
optimal conditions, the stability of the
oligonucleotide-duplex DNA interaction generally increases
continuously with oligonucleotide length. In the
preferred embodiment, a DNA oligonucleotide in the range
20 of about 20 to 40 bases is used. Oligonucleotides in this
range usually have useful dissociation constants for their
specific DNA target. The dissociation constants are in
the range of about 10^{-9} to 10^{-8} molar.
Oligonucleotides shorter than 20 bases display weaker and
25 less specific binding to the target sequence and are thus
less useful.

 Oligonucleotide binding to duplex DNA is
stabilized by binding to the purines in the underlying
30 duplex. Once a DNA target has been identified, the more
purine rich strand of the target area is defined as the
"orienting" strand of the binding site. An
oligonucleotide ligand was designed to bind either
parallel or anti-parallel to the orienting strand. The

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1 stability of the binding is dependent on the size of the
oligonucleotide and the location in the genome. Sometimes
the parallel is more stable than the anti-parallel while
at other times the reverse is true or they are equally
5 stable. In the preferred embodiment the method of
designing a detailed sequence of an oligonucleotide ligand
involves placing a T in the oligonucleotide whenever an AT
base pair occurs in the duplex target, and placing a G in
the oligonucleotide whenever a GC base pair occurs in the
10 duplex target.

Examples of the orientation of bond donors and
acceptors based on this oligonucleotide structure is
displayed in Figures 2 and 3.

15

Another embodiment of the present invention
includes a synthetic oligonucleotide for forming a
colinear triplex with a target sequence in a duplex DNA
when said target sequence is either about at least 65%
20 purine bases or about at least 65% pyrimidine bases,
comprising, a nucleotide sequence of at least about 20
nucleotides; said nucleotide sequence including G and T,
wherein G is used when the complementary location in the
duplex DNA is a GC base pair and T is used when the
25 complementary location in the duplex DNA is an AT base
pair; and said sequence selected from the group consisting
of an oligonucleotide oriented 3' to 5' and binding
anti-parallel to the about at least 65% purine strand in
the duplex DNA target sequence and an oligonucleotide
30 oriented 5' to 3' and binding parallel to the about at
least 65% purine strand in the duplex DNA target
sequence. Although molecules which include one or more
bases which do not comply with this relationship can be
fabricated, the binding affinity and site specificity of

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1 these altered oligonucleotides will be reduced.
Consequently the biological potency of these molecules
will be inferior to the oligonucleotides having the G/GC
and T/AT relationships.

5 Below is a schematic which demonstrates a target
sequence, and oligonucleotides ligands which have been
designed by the above design procedure.

10 Target Sequence (35bp)

5'-GGGAATTGGGCGGGTAATTTTCGGGATAGGCGGTAA-3'

3'-CCCTTAACCCGCCCATTAAGCCCTATCCGCCATT-5'

15 Parallel Synthetic Oligonucleotide

5'-GGGTTTTGGGGGGGTTTTTTGGGGTTTGGGGGTTT-3' (par)

Anti-Parallel Synthetic Oligonucleotide

20 3'-GGGTTTTGGGGGGGTTTTTTGGGGTTTGGGGGTTT-5' (anti)

If the synthetic oligonucleotide is constructed
with a standard phosphodiester linkage, its binding
25 affinity for the target would be near 10^{-7} M under
physiological conditions of salt, divalent ion
concentration and temperature. Since the dissociation
constant for oligonucleotide binding to a random DNA
sequence population is near 10^{-3} M for a 35 base
30 oligonucleotide, the synthetic oligonucleotide affinity
for the target would be approximately 10^4 times greater
than for random sequence DNA under the same conditions.

1 II. Analysis of the duplex target.

5 If these procedures are followed to make a
synthetic oligonucleotide, any duplex DNA sequence of
about at least 65% purines can form a stable triplex.
Within a DNA region, although the A+T content is not a
significant consideration, duplex DNA sequences which have
only purines on the template strand form complexes which
in general, are characterized by enhanced stability. If
10 we define n as the number of bases within the template
strand which are purine and define (1-n) as the number of
pyrimidine bases in the template, then the approximate
dissociation constant can be predicted from the following
semi-empirical formula:

15
$$K = \exp^{-[0.4n + (0.2(1-n)/RT)]}$$

 This formula assumes near-physiological
conditions in vitro, that is 0.05 M TRIS/HCl, 5mM MgCl₂,
20 3mM spermine pH 7.8, 37°C. These conditions constitute
the operating standard used in the design process.

 This relationship predicts that an
oligonucleotide designed to bind a 35 base long target
25 sequence containing only purine bases in its template
strand will form a triplex in which the oligonucleotide
binds with a standard dissociation constant of about
 1×10^{-10} M. This dissociation constant will be altered,
however, when pyrimidine is in the template strand. In
30 the above schematic representation where the template
contains pyrimidine, the dissociation constant is
 3×10^{-7} M.

1 This relationship is consistent with the
observation that the free energy of triplex formation
appears to increase in proportion to the span of the
target-oligonucleotide interaction and the observation
5 that the binding energy of a G to a GC base pair or a T to
an AT base pair is dependant on base pair orientation
relative to the template strand.

 The molecular origin of that effect can be seen
10 in Figure 2. It is evident that when the orienting strand
comprises a series of purines, the bases in the
complementary third strand form a contiguous stacked
array. On the other hand, placing a pyrimidine in the
orienting strand inverts the base pair. Thus, although
15 third strand hydrogen bonding can still occur with
parallel strand orientation upon forming a "Reverse
Hoogsteen" bond at the site of inversion, it is associated
with a dislocation of the path traversed by the third
strand in the major groove. Thus for either an AT or GC
20 base pair, approximately 0.4 kcal of favorable binding
free energy results from third strand association at a
purine site in the template, but only approximately 0.2
kcal when the third strand binds to a site at which a
purine to pyrimidine inversion has occurred.

25 III. Secondary chemical modification of the
oligonucleotide.

 A. One skilled in the art will recognize that a
30 variety of synthetic procedures are available. In the
preferred embodiment the oligonucleotides are synthesized
by the phosphoramidite method, thereby yielding standard
deoxyribonucleic acid oligomers.

1 Molecular modeling suggests that substitution of
the non-hydrolyzable phosphodiester backbone in the
oligonucleotide or elected sites may enhance the stability
of the resulting triplex in certain instances. The
5 phosphodiester analogues are more resistant to attack by
cellular nucleases. Examples of non-hydrolyzable
phosphodiester backbones are phosphorothioate,
phosphoroselenoate, methyl phosphate, phosphotriester and
the alpha enantiomer of naturally occurring
10 phosphodiester. The thiophosphate and methyl phosphonate
linkages are shown in Fig. 4. These non-hydrolyzable
derivatives of the proposed oligonucleotide sequences can
be produced, with little alteration of DNA target
specificity.

15 Backbone modification provides a practical tool
to "fine tune" the stability of oligonucleotide ligands
inside a living cell. For example, oligonucleotides
containing the natural phosphodiester linkage are degraded
20 over the course of 1-2 hours in eukaryotic cells, while
the non-hydrolyzable derivatives appear to be stable
indefinitely.

B. Oligonucleotide hybrids provide another
25 method to alter the characteristics of the synthetic
oligonucleotides. Linkers can be attached to the 5'
and/or 3' termini of the synthetic oligonucleotide. The
linkers which are attached to the 5' terminus are usually
selected from the group consisting of a base analogue with
30 a primary amine affixed to the base plane through an alkyl
linkage, a base analogue with a sulfhydryl affixed to the
base plane through an alkyl linkage, a long chain amine
coupled directly to the 5' hydroxyl group of the
oligonucleotide and a long chain thiol coupled directly to

1 the 5' hydroxyl group of the oligonucleotide. The linker
on the 3' terminus is usually a base analogue with a
primary amine affixed to the base plane through an alkyl
linkage or a base analogue with a sulfhydryl affixed to
5 the base plane through a alkyl linkage. Affixation of a
primary amine linkage to the terminus does not alter
oligonucleotide binding to the duplex DNA target.

Once a linkage has been attached to the synthetic
10 oligonucleotide a variety of modifying groups can be
attached to the synthetic oligonucleotide. The molecules
which can attach include intercalators, groove-binding
molecules, cationic amines or cationic polypeptides. The
modifying group can be selected for its ability to damage
15 DNA. For example, the modifying group could include
catalytic oxidants such as the iron-EDTA chelate, nitrogen
mustards, alkylators, photochemical crosslinkers such as
psoralin, photochemical sensitizers of singlet oxygen such
as eosin, methylene blue, acridine orange and 9 amino
20 acridine and reagents of direct photochemical damage such
as ethidium and various pyrene derivatives.

For example an "aminolink", as supplied by
Milligen (see Figure 5) works nicely. However, terminal
25 coupling of any sort is likely to be equivalent. Once
synthesized with an aminolink, the modified
oligonucleotides can be coupled to any reagent which is
specific for a primary amine, for example a succimide or
isothiocyanate moiety (Fig. 5).

30 In one embodiment, an "aminolink" coupling is
used to affix the intercalating dyestuff 9 acridine
isothiocanate to triplex forming oligonucleotides. The
duplex binding affinity of the oligonucleotide-dye hybrid

35

1 is approximately 100-fold greater than the oligonucleotide
binding affinity. Other embodiments include affixing
eosin isothiocyanate to oligonucleotides. Since eosin
isothiocyanate cleaves the DNA helix upon irradiation this
5 hybrid oligonucleotide cuts the helix at its binding site
when irradiated. This hybrid-oligonucleotide is useful
for identifying the oligonucleotide binding site both in
vitro and in vivo and potentially can be used as a
therapeutic tool for selective gene target destruction.

10

Photochemical reactivity is also achieved by
affixation of psoralin derivatives to oligonucleotides
through a 5' linkage. Psoralin binds covalently to DNA
after irradiation, and as a consequence is a potent
15 cytotoxic agent. Thus, photochemical reactivity, with
oligonucleotide sensitivity provides a tool to direct the
toxic psoralin lesion to the oligonucleotide target site.

Similar oligonucleotide coupling is used to
20 target toxic chemical reactivity to specific DNA
sequences. Examples include catalytic oxidants such as
transition metal chelates and nucleases.

Photochemical reactivity and/or toxic chemical
25 agents can be used to permanently inhibit gene expression.

In addition to chemical reactivity, modifications
of oligonucleotides alter the rate of cellular uptake of
the hybrid oligonucleotide molecules. The uptake process
30 is rapid, but poorly understood. Terminal modification
provides a useful procedure to modify cell type
specificity, pharmacokinetics, nuclear permeability, and
absolute cell uptake rate for oligonucleotide ligands.

35

1 C. Modified base analogues provide another means
of altering the characteristics of the synthetic
oligonucleotide. Although a purine rather than a
pyrimidine, X is identical to T with respect to its
5 capacity to form hydrogen bonds. Molecular modeling has
shown that substitution of X for T in the above
oligonucleotide design procedures, results in a modified
triplex that is much more stable. The increased stability
is due principally to enhanced stacking and to an
10 enhancement of phosphodiester backbone symmetry within the
ligand. Examples of base substitutions for T are X, I and
halogenated X and I. G can be replaced by halogenated G.
Furthermore, the 2' furanose position on the base can have
a non-charged bulky group substitution. Examples of
15 non-charged bulky groups include branched alkyls, sugars
and branched sugars. In the preferred embodiment at least
one base is substituted.

Molecular modeling suggests that oligonucleotide
20 design will produce ligands with target affinity and
specificity which exceeds that of even the most specific
antigen-monoclonal antibody interaction.

Synthetic oligonucleotides have been designed to
25 the transcription control region of the human c-myc
protooncogene, to the regulation sequence of collagen
I α , to bind to the TATA box segment of the chicken alpha
actin gene, and to bind to an enhancer sequence within the
early gene region of human HIV-I.

30 A further embodiment of the present invention is
a method of inhibiting the growth of cells, comprising the
step of administering a synthetic oligonucleotide in
sufficient amount for cellular uptake and binding to the
35

1 target sequence, wherein said target sequence is
positioned within the DNA domain adjacent to the RNA
transcription origin. The synthetic oligonucleotide is as
described above in the description of the design process.
5 Uptake into the cells is rapid for these synthetic
oligonucleotides and can be altered with the appropriate
substitutions and modifications. Similarly the binding
can be altered by appropriate changes to the synthetic
oligonucleotide. The inhibition of cell growth can be
10 used in the treatment of cancerous cells. Additions of
the specific oligonucleotide will selectively inhibit cell
growth. For example synthetic oligonucleotides to the
c-myc gene can be used to inhibit some cancerous cell
growth. Examples of synthetic oligonucleotide which
15 inhibit c-myc expression include: 3'-TGGTGTGTGGGTTTTGTGGG
GGGTGGGGGGGTTTTTTTTGGGTGGG-5' and/or
3'-TGTGGTGGGGTGGTTGGGGTGGGTGGGGTGGGTGGG-5' and/or
5'-TTTGGTGTGGGGGTGGGGGTTTTGTTTTTTGT-3' and/or
3'-GGTTGGGGTGGGTGGGGTGGGTGGGGT-5' and/or
20 5'-GGTTGGGGTGGGTGGGGTGGGTGGGGT-3' and fragments and
analogues thereof.

Another embodiment includes a method of
inhibiting the growth of pathogens comprising the step of
25 administering a synthetic oligonucleotide in sufficient
amount for cellular uptake and binding to the target
sequence, wherein said sequence binds within the nucleic
acid domain adjacent the RNA transcription origin. For
example HIV-1 virus can be inhibited with a synthetic
30 oligonucleotide which selectively binds to the viral LTR
region. Specific examples of this synthetic
oligonucleotide can include
3'-GTTTTTGGGTGTTGTGGGTGTGTGTGGTT-5' and/or
5'-TGGGTGGGGTGGGGTGGGGGGTGTGGGGTGTGGGGT-3' and fragments
35 and analogues thereof.

1 An additional embodiment includes a method of
manipulating the structural protein content of epidermal
tissue comprising the step of administering a synthetic
oligonucleotide in sufficient amount for cellular uptake
5 and binding to the target sequence. This includes
inhibiting the various enzymes and regulating proteins in
skin. For example, the collagen I α gene synthesis rate
can be altered by using
3'-TGGGTTGGGTGGTGGTGGGGGTGTGGTTTGGTTGTGGGTTTTT-5' and/or
10 3'-GTGGGTTGGGTGGTGGTGGGGGTGTGGTTTGG-5' and fragments and
analogues thereof as the synthetic oligonucleotide.
Similarly the collagenase gene can be inhibited by using
5'GGTTGGGGTTGGTGTGTTTTTTTTTGTGTGGGTG-3' and/or
15 5'-TTGTGGTTGTTTTTTTTTGGTTGTGTGTGT-3, and fragments and
analogues thereof.

 The following examples are offered by way of
illustration and are not intended to limit the invention
in any manner. The synthetic oligonucleotides described
20 in the examples can include any of the substitutions
discussed earlier. The backbone, base, linkers and
modifying groups can be added. These substitutions will
enhance the affinity, the chemical stability, and the
cellular uptake properties of the specific oligonucleotide
25 treatments.

Example 1.

A. A Method For Arresting the Growth of Cancerous
30 Tissue in Man, by Means of Intervention into the Program
of c-myc Gene Expression.

 Available evidence suggests that a family of
tumors, including Burkitt's lymphoma and others, share a
35

1 common genetic lesion, which is evident as constitutive
overproduction of the c-myc mRNA and its corresponding
c-myc protein. Because the c-myc protein has been shown
to be a critical element in the control of cell growth, it
5 is believed that there may be a direct causal relation
between the overproduction of c-myc protein and
uncontrolled cancerous growth for such cells.

In both cancerous and normal cells, the c-myc
10 gene possesses several target sequences within its 5'
flanking sequence which satisfy the synthetic
oligonucleotide design criteria. In a program of drug
development, these target sequences and others are used as
templates to direct oligonucleotide design. The purpose
15 of these oligonucleotides is to selectively inhibit c-myc
transcription, thereby repressing the uncontrolled growth
of tumors with the c-myc lesion.

Three representative target sequences in the
20 transcription control region of the human c-myc gene are
shown below:

A. TARGET: THE TATA BOX FOR THE C-MYC GENE

25 DNA TARGET DUPLEX

-61 -16
5'-TCCTCTCTCGCTAATCTCCGCCCACCGGCCCTTTATAATGCGAGGG-3'
3'-AGGAGAGAGCGATTAGAGGCGGGTGGCCGGGAAATATTACGCTCCC-5'

30 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TGGTGTGTGGGTTTTGTGGGGGGTGGGGGGTTTTTTTTTGGGTGGG-5'

1 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TGGTGTGTGGGTTTTGTGGGGGTGGGGGGGTTTTTTTTTGGGTGGG-3'

- 5 B. TARGET: TRANSCRIPTION ACTIVATOR BINDING SITE -
THE PRINCIPAL ACTIVATING PROTEIN BINDING SITE OF
THE C-MYC GENE PROMOTER

10 Inappropriately high levels of c-myc gene
expression are strongly associated with the incidence of a
variety of human tumors. The triplex oligonucleotides
described here were designed to selectively repress the
expression of the c-myc gene in such tumors, thereby
slowing tumor growth.

15

(1) DNA TARGET DUPLEX

-153

-116

5'-TCTCCTCCCCACCTTCCCCACCCTCCCCACCCTCCCCA-3'

20 3'-AGAGGAGGGGTGGAAGGGGTGGGAGGGGTGGGAGGGGT-5'

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE

5'-GTGGTGGGGTGGTTGGGGTGGGTGGGGTGGGTGGGGT-3

25

(2) DNA TARGET DUPLEX

-153

-116

5'-TCTCCTCCCCACCTTCCCCACCCTCCCCACCCTCCCCA-3'

30 3'-AGAGGAGGGGTGGAAGGGGTGGGAGGGGTGGGAGGGGT-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GTGGTGGGGTGGTTGGGGTGGGTGGGGTGGGTGGGT-5'

35

1 (3) DNA TARGET DUPLEX (27bp)

-142

-115

5'-CCTTCCCCACCCTCCCCACCCTCCCCA-3'

3'-GGAAGGGGTGGGAGGGGTGGGAGGGGT-5'

5

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GGTTGGGGTGGGTGGGGTGGGTGGGGT-5' (par)

10 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE

5'-GGTTGGGGTGGGTGGGGTGGGTGGGGT-3' (anti)

15

The 27 bp target duplex has 74% GC base pairs and 89%
purine on the orienting strand. The K_{diss} is $(6 \times 10^{-10} M)$
for anti-parallel binding.

C. TARGET: SEQUENCE BETWEEN TATA BOX AND ACTIVATOR
SITE IN A HIGHLY CONSERVED SEQUENCE AMONG THE VERTEBRATE
c-myc GENE FAMILY.

20

DNA TARGET DUPLEX

-87

-58

5'-AAAGCAGAGGGCGTGGGGGAAAAGAAAAAGA-3'

25 3'-TTTCGTCTCCCGCACCCCTTTTCTTTTTTCT-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TTTGGTGTGGGGGTGGGGGTTTTGTTTTTTGT-3'

30

ANTIPARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TTTGGTGTGGGGGTGGGGGTTTTGTTTTTTGT-5'

35

1 The likely function of these sites, the position relative
to the RNA transcription origin, and the oligonucleotide
sequence which can be used as a c-myc specific treatment
are shown. One skilled in the art will readily recognize
5 that as the molecular genetics of the c-myc gene is
elucidated in greater detail, the list of target sequences
within the 5' flanking region will be expanded, by
application of the above design criteria.

10 Both synthetic oligonucleotides A and B
specifically interact within the target duplex to inhibit
tumor growth, by means of specific repression of c-myc
transcription. The specific method of inhibition of
oligonucleotide C is unknown.

15 One skilled in the art will readily recognize
that oligonucleotides for other genes involved in human
tumors can be similarly designed. The procedure is only
limited by the available molecular sequence data.

20

Example 2.

A Method for Manipulating the Structural Protein
Content of Epidermal Tissues, for the Purpose of Altering
25 Tissue Appearance and Wound Healing.

The structural proteins which define the mechanical
properties of skin are well known. The molecular
structure of the collagen and elastin proteins and their
30 corresponding proteases, collagenase and elastase, have
been intensley studied. These proteins are under the
control of an elaborate program of regulation, which
appears to change during the wound healing process and as
a result of the aging process. The molecular structure is

35

1 sufficiently defined to consider treatments based upon
gene-specific intervention into the pattern of structural
protein synthesis and/or enzymatic degradation.

5 Data suggest that the change in the mechanical
properties of skin which accompanies aging (wrinkling,
etc.) is due in part to an age-specific change in the
relative abundance of the collagens and other structural
10 proteins. Interference with the synthesis and/or
selective degradation of these proteins by drug treatment
can reestablish a distribution which approximates that of
younger tissue, and thus the effects of aging can be
partially reversed.

15 A program of synthetic oligonucleotide design,
based upon manipulation of collagen I synthesis in human
skin is described below. By altering the relative protein
concentrations the structure and mechanical properties of
skin can be altered. Thus the synthetic oligonucleotide
20 can be used as a therapeutic agent to alter the skin aging
process or to alter the wound healing process. One
skilled in the art will readily recognize that the
concepts can be extended to other collagens, to other skin
proteins and to their complementary proteases based upon
25 the availability of the necessary genetic data.

Representative target sequences in the
transcription control region of the human alpha 1(I)
collagen gene, the likely function of those sites, their
30 position relative to the RNA transcription origin, and the
synthetic oligonucleotide sequence designed for collagen
specific treatment as shown below. As the molecular
genetics of the collagen gene develops, the list of target
sequences within the 5' flanking region will be expanded.

35

1 A. TARGET: THE CAT BOX FOR THE COLLAGEN GENE

DNA TARGET DUPLEX

-168
-124

5' -TCCCTTCCTCCTCCCCCTCTCCATTCCAACTCCCAAATTT-3'
3'-AGGGAAGGGAGGAGGAGGGGGAGAGGTAAAGGTTGAGGGTTTTAA-5'

SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

10
3'-TGGGTTGGGTGGTGGTGGGGTGTGGTTTGGTTGTGGGTTTTT-5'

B. TARGET: ENHANCER FOR THE COLLAGEN GENE

15

DNA TARGET DUPLEX

20

-294	-264
5' -CCCTACCCACTGGTTAGCCACGCCATTCT-3'	
3' -GGGATGGGTGACCAATCGGGTGCGGTAAGA-5'	

SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GGGTTGGGTGTGGTTTGGGGTGGGGTTTGG-5'

C. TARGET: HIGHLY CONSERVED POLYPURINE SEGMENT WHICH OCCURS NEAR -200 IN ALL COLLAGENS

30 DNA TARGET DUPLEX

-177

-136

5' -CTCCCTTCCCTCCTCCTCCCCCTCTCCATTCC-3'

3' -GAGGGAAGGGAGGAGGAGGGGGGAGAGGTAAGG-5'

1 SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GTGGGTTGGGTGGTGGTGGGGGTGTGGTTTGG-5'

5 Synthetic oligonucleotides A and B inhibit type I collagen protein synthesis. The process includes the specific repression of collagen RNA transcription. The method of inhibition of the C synthetic oligonucleotide is not known. The effect on protein synthesis of skin
10 proteins can be seen by adding sufficient amounts of the synthetic oligonucleotide for uptake into cultured human fibroblasts.

15 Next, two representative target sequences are described in the transcription control region of the human collagenase gene, the function of these sites, their position relative to the RNA transcription origin, and the oligonucleotide sequence designed as a collagen specific
20 treatment. As the molecular genetics of the collagenase gene develops, the list of target sequences within the 5' flanking region will be expanded.

D. TARGET: THE TATA BOX FOR THE COLLAGENASE GENE

25 DNA TARGET DUPLEX

-48

-16

5'-GGAAGGGCAAGGACTCTATATATACAGAGGGAG-3'
30 3'-CCTTCCCGTTCCTGAGATATATATGTCTCCCTC-5'

SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-GGTTGGGGTTGGTGTGTTTTTTTTGTGTGGGTG-3'

35

1 E. TARGET: THE INDUCIBLE ENHANCER FOR THE
COLLAGENASE GENE. CONFIRMS TPA TUMOR PROMOTOR
RESPONSIVENESS

5 DNA TARGET DUPLEX

-91

-64

5'-AAGAGGATGTTATAAAGCATGAGTCAGA-3'

3'-TTCTCCTACAATATTTCTGTA~~CT~~CAGTCT-5'

10 SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TTGTGGTTGTTTTTTTGGTTGTGTGTGT-3'

15 The D synthetic oligonucleotide inhibits
collagenase protein synthesis. The process includes
specific repression of collagenase RNA transcription. The
E synthetic oligonucleotide causes loss of TPA
20 sensitivity, and a subsequent repression of collagenase
syntheses in the presence of promoters such as TPA. This
process includes specific repression of collagenase RNA
transcription. Synthetic oligonucleotide interaction will
cause collagen protein levels in the cell to rise, as
25 collagenase levels fall. The clinical effect of the
increase should cause a useful alteration of the
mechanical properties of skin. The effects can be seen by
adding sufficient amounts of oligonucleotide for cellular
uptake to cultured human fibroblasts.

30 One skilled in the art will readily appreciate
that these concepts can be extended to other genes which
are known to be involved in skin development, repair and
aging and is only limited by the available molecular
35 genetic data.

Example 3

1
A Method to Repress the Growth of Human HIV-1 Virus,
by means of Oligonucleotide Binding to Target Sites
5 within the HIV-1 LTR.

10 The HIV-I virus is known to be the causative agent in human acquired immune deficiency syndrome (AIDS). The long terminal repeat of the HIV-1 virus is known to possess several DNA segments within the LTR region which are required for transcription initiation in a human T-cell host. The synthetic oligonucleotides selectively repress HIV-1 mRNA synthesis in a human host cell, by means of triplex formation upon target sequences within the viral LTR. Repression of an RNA synthesis
15 results in the reduction of the growth rate of the virus. This could result in the slowing of the infection process or the repression of the transition from latency to virulent growth. Most of the sites within the LTR will comprise target sites for drug (oligonucleotide)
20 intervention. There is no wasted DNA in the small, highly conserved LTR region.

25 Representative target sequences in the transcription control region of the human HIV-1 LTR, the likely function of these sites, their position relative to the RNA transcription origin, and the oligonucleotide sequence designed as a HIV-I specific treatment are shown below. As the molecular genetics of HIV-I develops, the
30 list of target sequences within the LTR and elsewhere will be expanded.

1 In all instances, both the parallel and
antiparallel isomers are described. The reason is that,
although one or the other will always display the better
binding affinity in vitro, the efficacy of each must be
5 tested in vivo to make the final decision.

A. TARGET: THE 5' END OF THE HIV-1 LTR DOMAIN

DNA Target Duplex (25bp, 92% Purine)

10 -470 -446
5'-AAAAGAAAAGGGGGGACTGGAAGGG-3'
3'-TTTCTTTTCCCCCTGACCTTCCC-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 5'-TTTTGTTTTGGGGGGTGTGGTTGGG-5' (HIV1par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

20 3'-TTTTGTTTTGGGGGGTGTGGTTGGG-5' (HIV1anti)

B. TARGET SITE: A segment of the negative HIV1
regulatory domain, with similarity to a homologous domain
in interleukin 2 gene.

25 DNA Target Duplex (33bp, 88% purine)

 -293 -261
5'-AGAGAAGGTAGAAGAGGCCAATGAAGGAGAGAA-3'
3'-TCTCTTCCATCTTCTCCGGTTACTTCCTCTCTT-5'

30 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TGTGTTGGTTGTTGTGGGGTTTGTGTTGGTGTGTT-3' (HIV2par)

35

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TGTGTTGGTTGTTGTGGGGTTTGTTCGGTGTGTT-5' (HIV2anti)

5 C: TARGET SITE: A site near the center of the LTR.

DNA Target Duplex (25bp, 88% purine)

10

-229	-205
9327	9351
5' -GGGATGGAGGACGCGGAGAAAGAAG-3'	
3' -CCCTACCTCCTGCGCCTCTTTCTTC-5'	

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 5'-GGGTTGGTGGTGGGGGTGTTTGTTG-3' (HIV3par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

20 3'-GGGTTGGTGGTGGGGGTGTTTGTG-5' (HIV3anti)

D. TARGET SITE:

25 Binding site for the Spl-line transcription
activator.

(1) DNA Target Duplex (36bp, 78% purine)

-80 -51

30 5' -AGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGCG-3'

3' -TCCCTCCGCACCGGACCCGCCCTGACCCCTCACCGC-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

1 5'-TGGGTGGGGTGGGGTGGGGGGGTGTGGGGTGTGGGG-3' (HIV4par) or
(HIV36par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5 3'-TGGGTGGGGTGGGGTGGGGGGGTGTGCCCTCTGGGG-5' (HIV4anti) or
(HIV36anti)

10 The HIV4 par also functions if TG is added to the 3' end
to make HIV38 par.

E. TARGET: BINDING SITE FOR THE TRANSCRIPTION
ACTIVATOR REGION (tar); THE DOWNSTREAM HALF OF THE tar SITE

15 DNA TARGET DUPLEX (29-31bp, 72% purine)

-16

+13

5'-CTTTTGCCTGTACTGGGTCTCTCTGGTTAG-3'

3'-GAAAAACGGACATGACCCAGAGAGACCAATC-5'

20 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GTTTTTGGGTGTTGTGGGTGTGTGGTT-5' (HIV29par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

25 5'-GTTTTTGGGTGTTGTGGGTGTGTGGTTT-3' (HIV31anti.)

30 The oligonucleotides, HIV29par and HIV31anti, were
designed as previously described herein. HIV31anti also
functions if bases two TG are removed from the 3' end.
The relative mobility and DNA footprint analyses of both
oligonucleotides show binding with high affinity to target
proviral sequences, in vitro.

35

1 HIV-1 infected U937 cells, a monocytoid line,
were treated with up to 20 μ M with either HIV29par,
HIV31anti, or a random isomer of HIV29 with no detectable
in vitro affinity for the target sequence. Significant
5 inhibition of viral mRNA production, as shown by the
decrease in the relative concentrations of env as compared
to β -actin mRNA, was achieved at a dose of 10 μ M of
either oligonucleotide ($p < .01$, paired t-test, figure
6). No additional suppression was observed at 20 μ M.
10 The random isomer of HIV29 did not inhibit viral mRNA
synthesis, even at 20 μ M, confirming the specificity of
the suppression achieved with HIV29.

We found that when U937/HIV-1 cells were
15 incubated in media containing 0.6 μ M 32 P-labeled
HIV29par, the cells were able to rapidly sequester the
oligomer in concentrations exceeding that of the media.
Assuming an average cell volume of 350 fL, it was
determined that the intracellular concentration increased
20 from 2.4 μ M after 10 minutes to a plateau of about 6 μ M
after 2 hours. The oligonucleotides had a prolonged
effect on HIV-1 transcription in that two treatments,
spaced two hours apart, inhibited viral mRNA synthesis for
up to 72 hrs (figure 7). Further studies showed the
effect of tar sequence specific oligonucleotides on
25 infected T cells. HIV29par was used to treat HIV-infected
H9 T cells. Treatment every 2 hrs. with 5 μ M effectively
suppressed mRNA synthesis in HIV-1 infected H9 T cells at
2 and 12 hours.

30 Thus, the evidence shows that the
oligonucleotides designed to bind within the major groove
of the DNA helix, and form triplexes with specific gene
sequences in the tar region of the HIV-1 provirus are

1 readily taken up by HIV-1 infected cells and selectively
suppress synthesis of HIV-1 mRNA without concomitant
suppression of mRNA for β -actin, which constitutive
expressed in these cells. With inhibition of viral MNRA
5 synthesis, translation of virus-encoded proteins is also
suppressed. Inhibition of viral mRNA depended on the dose
of oligonucleotide added; maximum inhibition occurred at
concentrations $\geq 10\mu\text{M}$. The oligonucleotides designed
to bind to specific sequences in the DNA duplex and form
10 colinear triplex with the targeted sequences provide an
efficient and highly specific agent for regulating gene
expression, such agents provide a new class of rationally
designed chemotherapeutic agents for controlling virus
replication and other processes depend upon new mRNA
15 production.

The synthetic oligonucleotides in A through E
will inhibit HIV-I mRNA synthesis, hence viral growth.
The process includes specific repression of RNA
20 transcription from the viral LTR.

One skilled in the art will readily recognize
that these concepts can be extended to other genes which
are known to be involved in the infection process by which
25 HIV-I and other viruses act.

Example 4

A Method for Altering Chicken Skeletal Actin
30 Transcription.

A representative target sequence in the
transcriptions control region of the chicken skeletal
alpha actin gene, the function of that site, its position
35

1 relative to the RNA transcription origin, and the
oligonucleotide sequence which would be designed as an
actin specific treatment are shown below. As the
molecular genetics of the actin gene develops, the list of
5 target sequences within the actin control region will be
expanded.

A. TARGET: THE TATA BOX FOR THE CHICKEN SKELETAL
ALPHA ACTIN GENE

10

DNA TARGET DUPLEX

-30

-4

5' - GATAAAAGGCTCCGGGGCCGGCGGCGG-3'

3' - CTATTTTCCGAGGCCCCGGCCGCCGCC-5'

15

SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5' - GTTTTTTGGGTGGGGGGGGGGGGGGG-3'

20

This synthetic oligonucleotide molecule inhibits
actin protein sythesis, by specific repression of RNA
transcription. This inhibition can be assessed in
cultured chicken myoblasts. The intact chicken will show
a change in the quality of actin and other muscle proteins
whose synthesis if strongly coupled to actin expression.
25 The practical result of this change will be an alteration
of the properties of chicken meat.

30

One skilled in the art will readily appreciate
that these concepts can be extended to other genes which
are known to be involved in muscle growth and development,
and is limited by the available molecular genetic data.

35

Example 5

INTERLEUKIN 2 ALPHA CHAIN RECEPTOR

TARGET: TRANS PROMOTOR REGION

DNA Target Duplex (28bp)

-273

-246

5'-AACGGCAGGGGAATCTCCCTCTCCTTTT-3'

3'-TTGCCGTCCCCTTAGAGGGAGAGGAAAA-5'

Parallel Synthetic Oligonucleotide

5'-TTGGGGTGGGGTTTGTGGGTGTGGTTTT-3' (IL28par)

Anti-Parallel Synthetic Oligonucleotide

3'-TTGGGGTGGGGTTTGTGGGTGTGGTTTT-5' (IL28anti)

The 28bp target is comprised of 54% G+C base pairs and is 61% purine on the orienting strand. The Kdiss for the parallel stand is 1.5×10^{-7} and the Kdiss for the antiparallel is 8×10^{-7} .

Example 6

A Sequence For Dispersing Plaque Formation
in Alzheimers Disease

The APP770 Gene is the precursor protein responsible for production of plaque in Alzheimers disease.

A. TARGET SITE: DOWNSTREAM TATA BOX SITE

DNA Duplex Target

-712-679

5 5' -AAAAACAAACA AAAATATAAGAAAGAAA CAAA-3'

 3'-TTTTTGTTTGT TTTTATATTCTTTC TTGTTTT-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

10 5'-TTTTGTTTGTTTTTTTTTCTTCTTTCTTTT-3' (APPlpar)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 3'-TTTTGTTGTTTTTTTTCTTCTTTCTTT-5' (APPlanti)

B. TARGET: UNKNOWN

20 DNA Duplex Target

5' -TCCTGCGCCTTGCTCCTTTGGTTCGTTCT-3'
3' -AGGACGCGGAACGAGGAAACCAAGCAAGA-5'

25
PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TGGTGGGGGTTGGTGGTTTGGTGGTTGT-5' (APP2par)

30 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TGGTGGGGGTTGGTGGTTTGGTTGGTTGT-3' (APP2anti)

35

1 C. TARGET: UNKNOWN

DNA Duplex Target

5 -477 -440
5'-TTCTCATTCTCTTCCAGAAACGCCTGCCCCACCTCTCC-3'
3'-AAGAGTAAGAGAAGGTCTTTGCGGACGGGGTGGATAGG-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

10 3'-TTGTGTTTGTGTTGGTGTGTTGGGGTGGGGGTGGTGTGG-5' (APP3par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 5'-TTGTGTTTGTGTTGGTGTGTTGGGGTGGGGGTGGTGTGG-3' (APP3anti)

D. TARGET: UNKNOWN

20 DNA Duplex Target

-434 -407
5'-GAGAGAAAAAACGAAATGCGGATAAAAA-3'
3'-CTCTCTTTTTTGCTTTACGCCTATTTTT-5'

25 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-GTGTGTTTTTTGGTTTTGGGGTTTTTTTT-3' (APP4par)

30 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GTGTGTTTTTTGGTTTTGGGGTTTTTTTT-5' (APP4anti)

35

1 E. TARGET: UNKNOWN

DNA Duplex Target

5 -286 -252
5'-CTCACCTTTCCCTGATCCTGCACCGTCCCTCTCCT-3'
3'-GAGTGGAAAGGGACTAGGACGTGGCAGGGAGAGGA-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

10 3'-GTGTGGTTTGGGTGTTGGTGGTGGGTGGGTGTGGT-5' (APP5par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 5'-GTGTGGTTTGGGTGTTGGTGGTGGGTGGGTGTGGT-3' (APP5anti)

F. TARGET: UNKNOWN

20 DNA Duplex Target

-264 -230
5'-CCGTCCCTCTCCTGGCCCCAGACTCTCCCTCCC-3'
3'-GGCAGGGAGAGGACCGGGGTCTGAGAGGGAGGG-5'

25 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GGGTGGGTGTGGTGGGGGTGTGTGTGGGTGGG-5' (APP6par)

30 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-GGGTGGGTGTGGTGGGGGTGTGTGTGGGTGGG-3' (APP6anti)

35

1 G. TARGET: UNKNOWN

DNA Duplex Target

5 -200 -177
5'-GGGGAGCGGAGGGGGCGCGTGGGG-3'
3'-CCCCTCGCCTCCCCCGCGCACCC-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

10 5'-GGGGTGGGGTGGGGGGGGTGGGG-3' (APP7par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 3'-GGGGTGGGGTGGGGGGGGTGGGG-5' (APP7anti)

H. TARGET: UNKNOWN

20 DNA Duplex Target

-40 -9
5'-CTCGCCTGGCTCTGAGCCCCGCCGCCGCTC-3'
3'-GAGCGGACCGAGACTCGGGGCGGCGGCGGAG-5'

25 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GTGGGGTGGGTGTGTGGGGGGGGGGGGGGGTG-5' (APP8par)

30 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-GTGGGGTGGGTGTGTGGGGGGGGGGGGGGGTG-3' (APP8anti)

35

Example 7

THE EGFR PROMOTOR DOMAIN

1
5 Inappropriately high expression of the epidermal growth factor gene (EGFR) has been implicated as crucial to the development of cancers and several skin diseases (psoriasis). The synthetic oligonucleotides described below were designed to selectively repress the expression of the EGFR gene in such diseases.
10

A. TARGET: SP1 BINDING SITE

15 DNA Duplex Target

-109 -83
5'-TCCGCCGAGTCCCCGCCTCGCCGCC-3'
3'-AGGCGGCTCAGGGGCGGAGCGGCGG-5'

20 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TGGGGGGTGTGGGGGGGTGGGGGGG-5' (EGFRlpar)

25 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TGGGGGGTGTGGGGGGGTGGGGGGG-3' (EGFRlanti)

30

35

1 D. TARGET: NUCLEASE SENSITIVE DOMAIN REQUIRED FOR
EGFR EXPRESSION

DNA Duplex Target

5

-363

-338

5'-TTCTCCTCCCTCCTCCTCGCATTCTCCTCCTCCTCT-3'

3'-AAGAGGAGGGAGGAGGAGCGTAAGAGGAGGAGGAGA-5'

10

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TTGTGGTGGGTGGTGGTGGGTGGGTGGTGGTGGTGT-5' (EGFR4par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15

5'-TTGTGGTGGGTGGTGGTGGGTGGGTGGTGGTGGTGT-3' (EGFR4anti)

Example 8

20

THE GSTpi GENE

Overexpression of the enzyme

glutathione-s-transferase pi has been implicated as being
25 responsible for the broad-range drug resistance which
developes in a variety of cancers. The synthetic
oligonucleotides described below are designed to repress
GST-pi expression, thereby sensitizing cancerous tissue to
traditional drug chemotherapy.

30

A. TARGET SITE: The target domain comprizes the
consensus binding sequences for the transcription
activating factors AP1 and Sp1. Synthetic
Oligonucleotides targeted against this will repress GSTpi
35 transcription by means of competition with AP1 and Sp1.

1 DNA Duplex Target

-68

-39

5'-GACTCAGCACTGGGGCGGAGCGGGGCGGGA-3'

5 3'-CTGAGTCGTGACCCCGCCTCGCCCCGCCCT-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-GTGTGTGGTGTGGGGGGGTGGGGGGGGGGT-3' (GST1par)

10

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GTGTGTGGTGTGGGGGGGTGGGGGGGGGGT-5' (GST1anti)

15 B. TARGET SITE: An enhancer-like polypurine sequence. A synthetic oligonucleotide targeted against this site will repress GSTpi transcription by means of competition with the enhancer.

-227

-204

20 5'-GGGGACCTGGGAAAGAGGGAAAGG-3'

3'-CCCCTGGACCCTTTCTCCCTTTCC-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

25

5'-GGGGTGGTGGGTTTGTGGGTTTGG-3' (GST2par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

30 3'-GGGGTGGTGGGTTTGTGGGTTTGG-5' (GST2anti)

An unusual repetitive DNA segment. No function has been ascribed to this segment yet. However, it is within the control domain and may play a role in transcription initiation.

35

1 DNA Duplex Target

-499 -410

5' -AAAATAAAATAAAATAAAATAAAAT-3'

3' -TTTTATTTTATTTTATTTTATTTTATTTTA-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3' (GST3par)

10 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-5' (GST3anti)

15 Example 9

The HMGCoA REDUCTASE GENE

HMGCoA Reductase is the enzyme which defines the rate limiting step in cholesterol biosynthesis. Its molecular genetics has been studied to understand the control of cholesterol synthesis. The described synthetic oligonucleotides will intervene in the program of cholesterol synthesis by means of modulating the transcription of HMGCoA.

A. TARGET SITE: The target is binding site for a repressor protein that appears to mediate end-product inhibition of transcription by cholesterol. The synthetic oligonucleotide is a synthetic repressor of HMGCoA expression, as an agonist of the cellular repressor.

1 DNA Duplex Target

-167

-135

5'-GGTGAGAGATGGTGCGGTGCCCCGTTCTCCGCCC-3'

5 3'-CCACTCTCTACCACGCCACGGGCAAGAGGCGGG-5

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GGTGTGTGTTGGTGGGGTGGGGGTTGTGGGGGG-5' (HMGCOA₁par)

10

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-GGTGTGTGTTGGTGGGGTGGGGGTTGTGGGGGG-3' (HMGCOA₁anti)

15

B. TARGET SITE: The target is a binding site for protein that appears to activate transcription of HMGCoA. The synthetic oligonucleotide against this site is a synthetic repressor of HMGCoA expression, as an antagonist of the cellular protein which binds to the target.

20

DNA Duplex Target

-134

-104

5'-GGGTGCGAGCAGTGGGCGGTTGTTAAGGCCA-3'

25 3'-CCCACGCTCGTCACCCGCCAACAATCCGCT-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-GGGTGGGTGGTGTGGGGGGTTGTTTTGGGGT-3' (HMGCOA₂par)

30

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GGGTGGGTGGTGTGGGGGGTTGTTTTGGGGT-5' (HMGCOA₂anti)

35

C. TARGET SITE: The target is a binding site for a protein that appears to activate transcription of HMGC_oA by binding to the "TATA box" domain. A TFO against this site is designed to be a synthetic repressor of HMGC_oA expression, as an antagonist of the cellular protein which binds to the TATA box target.

DNA Duplex Target

-41-6

10 5' -AGGCGATCGGACGATCCTTTCTTATTGGCGGCCCT-3'

 3' -TCCGCTAGCCTGCTAGGAAGAATAACCGCCGGGA-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 3'-TGGGGTTGGGTGGTTGGTTTGTTTTTGGGGGGGGT-5' (HMGOA3par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

20 5'-TGGGGTTGGGTGGTTGGTTTGTTTTGGGGGGGGT-3' (HMGCOA3anti)

Example 10

Nerve Growth Receptor (NGFR)

25 The NGFR gene encodes a cell surface receptor
required for nerve cell proliferation. It is
overexpressed in neuroblastoma and melanomas. Triplex
oligonucleotides are designed to repress the growth of
those cancerous tissues. Activation of the gene would be
a precondition of activation of nerve cell regeneration.
30 The mRNA start site is at -122 in this number scheme.

1 A. TARGET SITE: Consensus Spl binding site

DNA Duplex Target

5 -323 -290
5'-GGGAACTGGGTACCAGGGCGGGATGGGTGAGAGG-3'
3'-CCCTTGACCCATGGTCCCGCCCTACCCACTCTCC-5'

10 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-GGGTTGTGGGTGGTGGGGGGGTTGGGTGTGTGG-3'
(NGFR1par)

15 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-GGGTTGTGGGTGGTGGGGGGGTTGGGTGTGTGG-5' NGFR1ap

20 B. TARGET SITE: Consensus SP1 binding site.

DNA Duplex Target

25 -309 -275
5'-AGGGCGGGATGGGTGAGAGGCTCTAAGGGACAAGG-3'
3'-TCCCGCCCTACCCACTCTCCGAGATTCCCTGTTCC-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

30 5'-TGGGGGGGTTGGGTGTGTGGGTGTTTGGGTGTTGG-3'
(NGFR2par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

35 3'-TGGGGGGGTTGGGTGTGTGGGTGTTTGGGTGTTGG-5'
(NGFR2anti)

1 C. TARGET SITE: Domain flanking consensus Spl
binding sites.

DNA Duplex Target

5

-285

-248

5'-AAGGGACAAGGCAGGGAGAAGCGCACGGGTGCGGGAA-3'

3'-TTCCCTGTTCCGTCCCTCTTCGCGTGCCCACGCCCTT-5'

10

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TTGGGTGTTGGGTGGGTGTTGGGGTGGGGTGGGGGTT-3'
(NGFR3par)

15

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TTGGGTGTTGGGTGGGTGTTGGGGTGGGGTGGGGGTT-5'
(NGFR3anti)

20

D. TARGET SITE: Domain flanking consensus Spl
binding sites.

DNA Duplex Target

25

-243

-216

5'-CCCTCCCTTTGCCTCTGCTTCCCACCCC-3'

3'-GGGAGGGAAACGGAGACGAAGGGTGGGG-5'

30

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-GGGTGGGTTTGGGTGTGGTTGGGTGGGG-3' (NGFR4par)

35

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

1 3'-GGGTGGGTTTGGGTGTGGTTGGGTGGGG-5' (NGFR4anti)

TARGET SITE: Consensus Spl binding site.

5 DNA Duplex Target

-187

-154

5'-GGGGGTGGGCGGGCTGGCGGGGCGGAGGCGGGGG-3'

10 3'-CCCCACCCGCCGACCGCCCCGCCTCCGCCCCC-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-GGGGGTGGGGGGGGTGGGGGGGGGTGGGGGGGG-3'
15 (NGFR5par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

20 3'-GGGGGTGGGGGGGGTGGGGGGGGGTGGGGGGGG-5'
(NGFR5anti)

Example 11

25 HERPES SIMPLEX VIRUS 1: DNA Polymerase and DNA binding
proteins

30 HSV-1 is responsible for a variety of skin
lesions and other infections. The triplex oligonucleotide
are designed to bind directly to the promotor region of
the genes which encode the viral DNA polymerase and DNA
binding protein, thereby arresting viral replication.
Both genes occur at 0.4 map units and flank the
replication origin oriL. Numbering below is in terms of
the polypeptide start site for each gene.

35

1 A. TARGET SITE This site is in the 5' flanking
sequence of the DNA polymerase gene. The Angelotti strain
has three base changes relative to strain 17.

5 (1) Strain 17

DNA Duplex Target

-60-26

10 5' - TTTTCTCTTCCCCCTCCCACATTCCCCTCTTT - 3'

3'-AAAAAGAGAAGGGGGAGGGGTGTAAGGGGAGAAA-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TTTTTGTGTTGGGGGTGGGGGTGTGGGGGTGTTT-5'
(HSPOL17par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TTTTTGTGTTGGGGGGGTGGGGTGTGGGGGGTGTTT-3'
(HSVPOL17anti)

(2) Strain Angelotti

-62-26

25
5' - TTTTCTCTTCCCCCCTCCCCACATCCCCCCTCTTT - 3'

3'-AAAAAGAGAAGGGGGGGAGGGGTGTAGGGGGGAGAAA-5'

30 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TTTTTGTGTTGGGGGGGTGGGGTGTGGGGGGTGTTC-5'
(HSVPOL1par)

35 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

1 5--TTTTTGTGTTGGGGGGGTGGGGTGTGGGGGGTGT-3'
(HSVPOllanti)

5 A. TARGET SITE: This site is in the 5' flanking
sequence of the DNA binding protein gene for
strain 17.

-82 -118
5'-AAAATCCGGGGGGGGGGCGGCGACGGTCAAGGGGAGGG-3'
10 3'-TTTtaggccccccccccgCCGCTGCCAGTTCCTCCC-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 5'-TTTTTGGGGGGGGGGGGGGGGTGGGTGTTGGGGTGGG-3'
(HSVPOL2par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

20 3'-TTTTTGGGGGGGGGGGGGGGGTGGGTGTTGGGGTGGG-5'
(HSVPOL2anti)

Example 12

25 HERPES SIMPLEX VIRUS 1: origin of replication

HSV-1 is responsible for a variety of skin
lesions and other infections. The triplex
oligonucleotides are designed to bind directly to the two
30 classes of HSV-1 DNA replication origin, thereby arresting
viral replication. The first origin (oriL) occurs at 0.4
map units and is in between and immediately adjacent to
the HSV-1 DNA polymerase and DNA binding protein genes.
The two identical origins of the second type (oriS) occur

35

1 at 0.82 and 0.97 map units. Numbering below is the terms
of position relative to the two fold symmetry axis of each
origin.

5 A. TARGET SITE oriL origin

1. DNA Duplex Target

-48 -10
10 5'-AGGACAAAGTGCGAACGCTTCGCGTTCTCACTTTTTTT-3'
3'-TTTTTTTCACTCTTGCGCTTCGCAAGCGTGAAACAGGA-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

15 5'-TTTTTTTGTGTGTTGGGGTTGGGTGGGTGTTTGTGGT-3'
(HSVORL1par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

20 3'-TTTTTTTGTGTGTTGGGGTTGGGTGGGTGTTTGTGGT-5'
(HSVORL1anti)

2. DNA Duplex Target

25 10 47
5'-AGGACAAAGTGCGAACGCTTCGCGTTCTCACTTTTTTT-3'
3'-TCCTCTTTCTCGCTTGCGAAGCGCAAGAGTGAAAAAAA-5'

30 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TGGTGTGTTGTGGGTGGGTGGGTGTTGTGTGTTTTTT-5'
(HSVORL2par)

35

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TGGTGTGTTGTGGGTGGGTTGGGGTTGTGTGTTTTTTT-3'
(HSVORL2anti)

These two target sites are within the oriL origin. Because the oriL also comprises the 5' flanking domain of the HSV-1 DNA polymerase and the HSV-1 major DNA binding protein, these triplex oligonucleotides may also interfere with transcription of those two genes.

B. TARGET SITE: oriS origin

DNA Duplex Target

-69 -34
5'-AAGGGGGCGGGGCCCGGGTAAAAGAAGTGAGAA-3'
3'-TTCCCCCGCCCCGGCGGCCCATTTTCTTCACTCTT-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TTGGGGGGGGGGGGGGGGGGTTTGTGTGTGTT-3'
(HSVORS1par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TTGGGGGGGGGGGGGGGGGGTTTGTGTGTGTT-5'
(HSVORS1anti)

Example 13

HUMAN BETA GLOBIN

1
5 The beta globin gene encodes one of the proteins comprising adult hemoglobin. Mutation in this gene is responsible for beta thalassemia and sickle cell anemia. Triplex oligonucleotides targeted to this gene are designed to inhibit the beta globin gene in thalassemics and in patients with sickle cell anemia, to be replaced by the naturally occurring delta protein. Two classes of triplex oligonucleotides TFO are described, which are targeted against the 5' enhancer or the promotor/coding domain. Numbering is relative to the principal mRNA start site.
15

A. DNA Duplex Target

20 -912 -886
5'-CCTTTTCCCCTCCTACCCCTACTTTCT-3'
3'-GGAAAAGGGGAGGATGGGGATGAAAGA-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

25 3'-GGTTTGGGGTGGTTGGGGTTGTTTGT-5' (GL1par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

30 5'-GGTTTGGGGTGGTTGGGGTTGTTTGT-3' (GL1anti)

B. DNA Duplex Target

35 -63 -25

1 5'-AGGAGCAGGGAGGGCAGGAGCCAGGGCTGGGCATAAAAG-3'

3'-TCCTCGTCCCTCCCGTCCCTCGGTCCCCACCCGTATTTTC-5'

5 PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TGGTGGTGGGTGGGGTGGTGGGTGGGGTGGGGTTTTTTG-3'
(GL2par)

10 ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE)

3'-TGGTGGTGGGTGGGGTGGTGGGTGGGGTGGGGTTTTTTG-5'
(GL2anti

15 C. DNA Duplex Target

-36

-9

5'-AGGGCTGGGCATAAAAGTCAGGGCAGAG-3'

20 3'-TCCCGACCCGTATTTTCAGTCCCGTCTC-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5'-TGGGGTGGGGTTTTTTGTGTGGGGTGTG-3' (GL3par)

25

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

3'-TGGGGTGGGGTTTTTTGTGTGGGGTGTG-5' (GL3anti)

30 D. DNA Duplex Target

514

543

5'-CCCTTGATGTTTTCTTTCCCCTTCTTTTCT-3'

35

1

3'-GGGAAGTACAAAAGAAAGGGGAAGAAAAGA-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5

3'-GGGTTGTTGTTTTGTTTGGGGTTGTTTTGT-5' (GL4par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

10

5'-GGGTTGTTGTTTTGTTTGGGGTTGTTTTGT-3' (GL4anti)

E. DNA Duplex Target

693

719

15

5'-TTCTTGCTTTCTTTTTTTTTCTTCTCC-3'

3'-AAGAACGAAAGAAAAAAAAGAAGAGG-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

20

3'-TTGTTGGTTTGTTTTTTTTTTGTTGTGG-5' (GL5par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

25

5'-TTGTTGGTTTGTTTTTTTTTTGTTGTGG-3' (GL5anti)

F. DNA Duplex Target

874

900

30

5'-CTCCCTACTTTATTTCTTTTATTTT-3'

3'-GAGGGATGAAATAAAAGAAAATAAAAA-5'

PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

35

1 3'-GTGGGTTGTTTTTTTTTGTGTTTTTTTTT-5' (GL5par)

ANTI-PARALLEL SYNTHETIC OLIGONUCLEOTIDE SEQUENCE

5 5'-GTGGGTTGTTTTTTTTTGTGTTTTTTTTT-3' (GL6anti)

Example 14

10 Testing for the effect of oligonucleotide binding
in cells. The effects of triplex-forming oligonucleotides
are studied in cell culture. Oligonucleotides are
administered to cultured human cell lines, which are then
analyzed for oligonucleotide uptake and for a change in
15 the steady-state level of messenger RNA associated with
the DNA target. As an example, the methods for the c-myc
gene are shown. One skilled in the art will readily be
able to generalize to any gene within a cultured cell.

20 HeLa cells grown on a solid support (100ul total
volume), are treated with ³²P-labelled oligonucleotide,
then incubated as a function of time and concentration.
Cells are separated from serum by centrifugation and
exhaustive washing, are disrupted by deproteinization then
assayed quantitatively on a 8% sequencing gel. This
25 analysis procedure yields the following characteristics:

a. The apparent partition coefficient for
oligonucleotide uptake into HeLa cells.

30 b. The uptake rate, i.e., the time constant to
reach a steady state with respect to oligonucleotide
uptake.

1 c. The half-time for oligonucleotide
 degradation in serum and in the HeLa cell.

5 From those data, the optimized timecourse and
 titration range for the oligonucleotide treatment of cells
 is determined.

10 Transcription inhibition is assayed by a
 variation of the RNase protection assay, which is the
 standard assay for quantifying steady state mRNA levels in
 mammalian cells. Total cellular RNA is extracted from
 oligonucleotide-treated HeLa cells, then hybridized to a
 uniformly labelled antisense RNA transcript, generated by
 the action of T7 polymerase on the SmaI-PvuII human c-myc
15 frangment in pSPT19.

20 This SmaI-PvuII probe is complementary to the
 first myc exon and sequences which comprise both the P1
 and P2 transcription start sites of myc. When the probe
 is hybridized in excess over myc transcript, a limit
 RNaseI digest produces either a 0.6 kb duplex
 (transcription from P1, which is the preferred origin in
 HeLa cells) or a 0.4 kb duplex (transcription occurs
 instead from P2, which is used in HeLa cells under
25 conditions of serum starvation).

30 The size and quantity of the resulting RNase
 resistant duplexes is then determined by quantitative
 autoradiography on a 5% acrylamide gel matrix. This assay
 system can quantify steady-state RNA levels to within 20%
 accuracy, which is sufficient for the purposes of this
 analysis.

1 The outcome of these cellular titrations is
analyzed in the context of two control experiments. The
first is a comparison of the dose response of
oligonucleotides which bind selectively to the target gene
5 and the dose response of oligonucleotides which are
unrelated. If oligonucleotide-mediated repression of the
c-myc transcription is due to site-specific triplex
formation in the cell, then an unrelated oligonucleotide
will not elicit an affect, over an equivalent
10 concentration range.

 The second control addresses the gene specificity
of the effect. In the RNase protection assay, data are
always normalized to overall RNA concentration in the
15 cell. As such, changes in the steady state level of the
myc transcript are meaningful in their own right. However
to confirm that the effects of oligonucleotide binding are
specific to the c-myc gene we also assay for the effect of
myc-specific oligonucleotide treatment on the steady state
20 levels of the histone 2A (H2A) message in HeLa cells,
probing the RNA complement with an H2A antisense RNA,
generated from a construct which, as for myc sequences,
has been cloned into a RNA expression vector. When
oligonucleotide mediated repression is specific to the myc
25 gene, H2A transcription in HeLa cells will be unaffected,
over an equivalent concentration range.

 Over the 1 to 50 micro-molar range,
oligonucleotides which bind to the control region of the
30 human c-myc gene selectively repress c-myc transcription
in an intact HeLa cell. Preliminary work with other
oligonucleotides described in the examples have begun to
display similar selectivity.

1 One skilled in the art will recognize that
application of these methods is readily generalized to any
gene in any cell line and is limited only by the
availability of cloned gene constructs, DNA sequence data,
5 and a rudimentary understanding of the molecular genetics
of the gene under investigation. At present, that battery
of information is available for several hundred human
genes, and for several thousand genes from other species.

10 The methods can also be applied, without
significant modification to the use of chemically altered
oligonucleotides variants, such as those with chemical
moieties added to the 3' and 5' terminus, oligonucleotides
with an altered phosphodiester backbone or those with
15 bases other than G and T (i.e., iodo-G or X).

 Ultimately, the importance of these examples is
to show that a whole class of single strand
oligonucleotide molecules are readily taken up by
20 eukaryotic cells, without exogenous manipulation of any
kind. The uptake mechanism is not known at present, but
in most cells, it is efficient and, apparently,
independent of oligonucleotide sequence (Eppstein D.A.,
Schryver B.B. & Marsh Y.V. (1986) J. Biol.Chem. 261,
25 5999). Therefore, in the most general sense, the overall
uptake properties of such oligonucleotides are not
significantly different from other potent drugs. By this
criterion, it is certain that an oligonucleotide ligand
designed to selectively intervene into the process of gene
30 expression will show pharmacological effects in an intact
cell.

1 In the past, these cell uptake concepts have been
used to explain the effectiveness of RNA oligonucleotides
as drugs which enhance the effect of interferon treatment
(Eppstein D.A., Schryver B.B. & Marsh Y.V. (1986) J.
5 Biol.Chem. 261, 5999) and of the ability of "antisense" or
"anti-splice junction" oligonucleotides to selectively
inhibit mRNA processing in the cell (Heikkilä R. et. al.
(1987) Nature 328, 445 and Eppstein D.A., Schryver B.B. &
Marsh Y.V. (1986) J. Biol. Chem. 261, 5999). It is likely
10 that the same uptake process is the basis for the use of
triplex-forming oligonucleotides as drugs to selectively
regulate transcription initiation or to selectively
destroy a gene target.

15 The design process described herein can be used
to design a synthetic DNA oligonucleotide which will bind
specifically to any double strand DNA target of interest.
The resulting oligonucleotide-duplex DNA complex is best
described as a colinear triplex. In the triplex the
20 oligonucleotide molecule occupies the major groove of the
duplex. The complex is stabilized by base-base hydrogen
bonding at the surface of the major groove, leaving
Watson-Crick pairing intact. As a result, the stability
and site specificity of the synthetic oligonucleotide is
25 not significantly affected by modification of the
phosphodiester linkage or by chemical modification of the
oligonucleotide terminus. Consequently, these
oligonucleotides can be chemically modified; enhancing the
overall binding stability, increasing the stability with
30 respect to chemical degradation, increasing the rate at
which the oligonucleotides are transported into cells, and
conferring chemical reactivity to the molecules.

1 Based upon the design method described herein, it
is possible to design oligonucleotides which are readily
taken up by eukaryotic cells and, once in the cell, can be
targeted to specific sites within a genome. Currently,
5 the site specificity and stability of the synthetic
oligonucleotide-target site interaction is as good as
current monoclonal antibody-antigen binding interactions.

10 This new class of site specific molecules can be
used as gene-specific reagents with the capacity to
control the transcription process in a gene-specific
fashion. This control is effective on both somatic genes
and viral genes which have infected a host cell. When
synthetic oligonucleotides are appropriately coupled to a
15 reactive chemical complement, it is possible to create a
hybrid molecule with the capacity to selectively destroy a
gene target of interest.

20 One skilled in the art will readily appreciate
that the present invention is well adapted to carry out
the objects and attain the ends and advantages mentioned
as well as those inherent therein. The oligonucleotides,
compounds, methods, procedures and techniques described
herein are presently representative of preferred
25 embodiments, are intended to be exemplary, and are not
intended as limitations on the scope. Changes therein and
other uses will occur to those skilled in the art which
are encompassed within the spirit of the invention or
defined by the scope of the appended claims.

30

35

CLAIMS

1

1. A method for making a synthetic oligonucleotide which binds to a target sequence in duplex DNA forming a colinear triplex by binding to the major groove, said method comprising the steps of:

scanning genomic duplex DNA and identifying nucleotide target sequences of greater than about 20 nucleotides having either about at least 65% purine bases or about at least 65% pyrimidine bases; and

synthesizing said synthetic oligonucleotide complementary to said identified target sequence, said synthetic oligonucleotide having a G when the complementary location in the DNA duplex has a GC base pair, having a T when the complementary location in the DNA duplex has an AT base pair.

20

2. The method of Claim 1, wherein said synthetic oligonucleotide is selected from the group consisting of an oligonucleotide oriented 3' to 5' and binding anti-parallel to be about at least 65% purine strand and an oligonucleotide oriented 5' to 3' and binding parallel to the about at least 65% purine strand.

25

3. A synthetic oligonucleotide for forming a colinear triplex with a target sequence in a duplex DNA when said target sequence is either about at least 65% purine bases or about at least 65% pyrimidine bases, comprising,

30

a nucleotide sequence of at least about 20 nucleotides;

35

1

said nucleotide sequence including G and T, wherein G is used when the complementary location in the duplex DNA is a GC base pair and T is used when the complementary location in the duplex DNA is an AT base pair; and

5

10

said sequence selected from the group consisting of an oligonucleotide oriented 3' to 5' and binding anti-parallel to the about at least 65% purine strand in the duplex DNA target sequence and an oligonucleotide oriented 5' to 3' and binding parallel to the about at least 65% purine strand in the duplex DNA target sequence.

15

4. The synthetic oligonucleotide of claim 3, wherein, at least one T is replaced with a compound selected from the group consisting of X, halogenated derivatives of X, I and halogenated derivatives of I.

20

5. The synthetic oligonucleotide of claim 3, wherein, at least one G is replaced with a halogenated derivative of G.

25

6. The synthetic oligonucleotide of claim 3, wherein, at least one base is substituted at the 2' furanose position with a non-charged bulky group.

30

7. The synthetic oligonucleotide of claim 6, wherein, said non-charged bulky group is selected from the group consisting of a branched alkyl, a sugar and a branched sugar.

35

1 8. The synthetic oligonucleotide of claim 3,
wherein, the backbone is a phosphodiester analogue which
is not readily hydrolyzed by cellular nucleases.

5 9. The synthetic oligonucleotide of claim 8,
wherein, said phosphodiester analogue is selected from the
group consisting of phosphorothioate, phosphoroselenoate,
methyl phosphate, phosphoramidite, phosphotriester and the
alpha enantiomer of naturally occurring phosphodiester.

10 10. The synthetic oligonucleotide of claim 3,
further including a linker at a terminus.

15 11. The synthetic oligonucleotide of claim 10,
wherein, said linker is attached to the 3' terminus and is
selected from the group consisting of a base analogue with
a primary amine affixed to the base plane through an alkyl
linkage and a base analogue with a sulfhydryl affixed to
the base plane through an alkyl linkage.

20 12. The synthetic oligonucleotide of claim 10,
wherein said linker is attached to the 5' terminus and is
selected from the group consisting of a base analogue with
a primary amine affixed to the base plane through an alkyl
25 linkage, a base analogue with a sulfhydryl affixed to the
base plane through an alkyl linkage, a long chain amine
coupled directly to the 5' hydroxyl group of the
oligonucleotide and a long chain thiol coupled directly to
the 5' hydroxyl group of the oligonucleotide.

30 13. The synthetic oligonucleotide of claims 10,
11 or 12 further including a modifying group attached to
said linker, wherein, said modifying group binds to duplex
DNA and is selected from the group of molecules consisting

35

1 of an intercalator, a groove-binding molecule, a cationic
amine and a cationic polypeptide.

5 14. The synthetic oligonucleotide of claims 10,
11 or 12, further including a modifying group attached to
said linker, wherein said modifying group damages DNA and
is selected from the group of molecules consisting of a
catalytic oxidant, nitrogen mustard, alkylator,
photochemical crosslinker, photochemical sensitizer of
10 singlet oxygen and reagent capable of direct photochemical
damage.

15 15. The synthetic oligonucleotide of claim 14,
wherein said photochemical sensitizer of singlet oxygen is
eosin, methylene blue, acridine orange, or 9 amino
acridine.

20 16. The synthetic oligonucleotide of claim 14,
wherein said reagent is ethidium or pyrene derivatives.

25 17. A method of inhibiting the growth of cells,
comprising the step of administering the synthetic
oligonucleotide of claim 3 in sufficient amount for
cellular uptake and binding to the target sequence,
wherein said target sequence is positioned within the DNA
domain adjacent to the RNA transcription origin.

30 18. The method of claim 17, wherein the cells
are cancerous cells and the synthetic oligonucleotide is
specific to the C-myc gene.

1 19. The method of claim 18, wherein the
synthetic oligonucleotide is selected from the group
consisting of:

5 3'-TGGTGTGTGGGTTTTGTGGGGGGTGGGGGGTTTTTTTTGGGTGGG-5',
5'-TGGTGTGTGGGTTTTGTGGGGGGTGGGGGGTTTTTTTTGGGTGGG-3',
5'-GTGGTGGGGTGGTTGGGGTGGGTGGGGTGGGTGGGCT-3,
3'-GTGGTGGGGTGGTTGGGGTGGGTGGGGTGGGTGGGT-5',
3'-GGTTGGGGTGGGTGGGGTGGGTGGGGT-5',
10 5'-GGTTGGGGTGGGTGGGGTGGGTGGGCT-3',
3'-TTTGGTGTGGGGGTGGGGGTTTTGTTTTTTGT-5'
5'-TTTGGTGTGGGGGTGGGGGTTTTGTTTTTTGT-3' and fragments
and analogues thereof.

15 20. The method of claim 19, wherein, said
oligonucleotide includes a linker and modifying group.

20 21. A method of inhibiting the growth of pathogens
comprising the step of administering the synthetic
oligonucleotide of claim 3 in sufficient amount for cellular
uptake and binding to the target sequence, wherein said
sequence binds within the nucleic acid domain adjacent the RNA
transcription origin.

25 22. The method of claim 21, wherein the pathogen is
HIV-1 virus and the synthetic oligonucleotide is within the
viral LTR region.

30 23. The method of claim 22, wherein the synthetic
oligonucleotide is selected from the group consisting of:

5'-TTTTGTTTTGGGGGGTGTGGTTGGG-5',
3'-TTTTGTTTTGGGGGGTGTGGTTGGG-5',
5'-TGTGTTGTTGTTGTGGGGTTTGTGTTGTTGTT-3',

35

1 3'-TGTGTTGGTTGTTGTGGGGTTTGTGTTGGTGTGTT-5',
 5'-GGGTTGGTGGTGGGGGTGTTTGTG-3',
 3'-GGGTTGGTGGTGGGGGTGTTTGTG-5',
 3'-TGGGTGGGGTGGGGTGGGGGGGTGTGCCCTCTGGGG-5',
5 5'-GTTTTTGGGTGTTGTGGGTGTGTGTGGTTT-3',
 3'-GTTTTTGGGTGTTGTGGGTGTGTGTGGTT-5',
 5'-TGGGTGGGGTGGGGTGGGGGGGTGTGGGGTGTGGGGTG-3'
and fragments and analogues thereof.

10 24. The method of claim 23, wherein, said
oligonucleotide includes a linker and modifying group.

15 25. A method of manipulating the structural
protein content of epidermal tissue comprising the step of
administering the synthetic oligonucleotide of claim 3 in
sufficient amount for cellular uptake and binding to the
target sequence.

20 26. The method of claim 25 for inhibiting a
collagen gene, wherein the synthetic oligonucleotide is
selected from the group consisting of:

 3'-TGGGTGGGTGGTGGTGGGGGTGTGGTTTGGTTGTGGGTTTTT-5',
 3'-GGGTGGGTGTGGTTTGGGGTGGGGTTTGG-5',
25 3'-GTGGGTGGGTGGTGGTGGGGGTGTGGTTTGG-5' and
fragments and analogues thereof.

30 27. The method of claim 25, for inhibiting a
collagenase gene, wherein the synthetic oligonucleotide is
selected from the group consisting of

 5'GGTTGGGGTTGGTGTGTTTTTTTTTGTGTGGGTG-3',
 5'-TTGTGGTTGTTTTTTTGGTTGTGTGTGT-3'
and fragments and analogues thereof.

1

28. A method of permanently inhibiting gene expression comprising the step of administering the synthetic oligonucleotide of claim 14 in sufficient amount for cellular uptake and binding to the target sequence.

5

29. The method of claim 28, wherein the synthetic oligonucleotide is selected from the group consisting of eosin isothiocyanate, psoralin derivatives, metal chelates, ethidium and pyrene derivatives.

10

30. The method of altering the characteristics of muscle proteins in food animals comprising the step of administering the synthetic oligonucleotide of claim 3 in sufficient amount for cellular uptake and binding to the target sequence.

15

31. The method of claim 30, wherein the synthetic oligonucleotide is selected from the group consisting of:

20

5' - GTTTTTTGGGTGGGGGGGGGGGGGGGGG-3' and fragments and analogues thereof.

25

32. A method of inhibiting the interleukin 2 alpha chain receptor comprising the step of administering the synthetic oligonucleotide of claim 3 in sufficient amount for cellular uptake and binding to the target sequence.

30

33. The method of claim 32 for inhibiting interleukin 2 alpha chain receptor, wherein the synthetic oligonucleotide is selected from the group consisting of:

35

1 5'-TTGGGGTGGGGTTTGTGGGTGTGGTTTT-3',
 3'-TTGGGGTGGGGTTTGTGGGTGTGGTTTT-5'
and fragments and analogues thereof.

5 34. A method of disbursing plaque formation and
Alzheimer's Disease, comprising the step of administering
the synthetic oligonucleotide of claim 3 in sufficient
amount for cellular uptake and binding to the target
sequence.

10 35. The method of claim 34 for disbursing plaque
formation in Alzheimer's Disease, wherein the synthetic
oligonucleotide is selected from the group consisting of:

15 5'-TTTTTGTGTGTTTTTTTTTTCTTTCTTCTTTT-3',
 3'-TTTTTGTGTGTTTTTTTTTTCTTTCTTCTTTT-5',
 3'-TGGTGGGGGTGGTGGTTGGTTGGTTGT-5',
 5'-TGGTGGGGGTGGTGGTTGGTTGGTTGT-3',
 3'-TTGTGTTTGTGTTGGTGTGTTGGGGTGGGGGTGGTGTGG-5',
20 5'-TTGTGTTTGTGTTGGTGTGTTGGGGTGGGGGTGGTGTGG-3',
 5'-GTGTGTTTTTTTGGTTTTTGGGGTTTTTTTT-3',
 3'-GTGTGTTTTTTTGGTTTTTGGGGTTTTTTTT-5',
 3'-GTGTGGTTTGGGTGTTGGTGGTGGGTGGGTGTGGT-5',
 5'-GTGTGGTTTGGGTGTTGGTGGTGGGTGGGTGTGGT-3',
25 3'-GGGTGGGTGTGGTGGGGGGTGTGTGTGGGTGGG-5',
 5'-GGGTGGGTGTGGTGGGGGGTGTGTGTGGGTGGG-3',
 5'-GGGGTGGGGTGGGGGGGGTGGGG-3',
 3'-GGGGTGGGGTGGGGGGGGTGGGG-5',
 3'-GTGGGGTGGGTGTGTGGGGGGGGGGGGGGGTG-5',
30 5'-GTGGGGTGGGTGTGTGGGGGGGGGGGGGGGTG-3'
and fragments and analogues thereof.

1 36. The method of repressing the expression of
the epidermal growth factor gene comprising the step of
administering the synthetic oligonucleotide of claim 3 in
sufficient amount for cellular uptake and binding to the
5 target sequence.

37. The method of claim 36 for repressing
expression of the epidermal growth factor gene, wherein
the synthetic oligonucleotids is selected from the group
10 consisting of:

3'-TGGTGGGGGTTGGTGGTTTGGTGGTTGT-5',
5'-TGGTGGGGGTTGGTGGTTTGGTGGTTGT-3',
3'-TGGGTGGTGGTGGGGGGGTGGGTGGG-5',
15 5'-TGGGTGGTGGTGGGGGGGTGGGTGGG-3',
3'-TTGTGGTGGTGGTGTGGTGGTGGGGTTGGGTGGTGG-5',
5'-TTGTGGTGGTGGTGTGGTGGTGGGGTTGGGTGGTGG-3',
3'-TTGTGGTGGGTGGTGGTGGGTGGGTGGTGGTGGTGT-5',
5'-TTGTGGTGGGTGGTGGTGGGTGGGTGGTGGTGGTGT-3'

20 and fragments and analogues thereof.

38. A method of repressing the GSTpi gene
comprising the step of administering the synthetic
oligonucleotide of claim 3 in sufficient amount for
cellular uptake and binding to the target sequence.
25

39. The method of claim 38 for repressing the
GSTpi gene, wherein the synthetic oligonucleotide is
selected from the group consisting of:

30 5'-GTGTGTGGTGTGGGGGGGTGGGGGGGGGT-3',
3'-GTGTGTGGTGTGGGGGGGTGGGGGGGGGT-5',
5'-GGGGTGGTGGGTTTGTGGGTTTGG-3',
3'-GGGGTGGTGGGTTTGTGGGTTTGG-5',
35

1 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3',
 3'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-5'
and fragments and analogues thereof.

5 40. A method of intervening into the program of
cholesterol synthesis by modulating the transcription of
HMGCoA comprised in the step of administering the
synthetic oligonucleotide of claim 3 in sufficient amount
for cellular uptake and binding to the target sequence.

10 41. The method of claim 40 for modulating the
transcription of HMGCoA, wherein the synthetic
oligonucleotide is selected from the group consisting of:

15 3'-GGTGTGTGTTGGTGGGGTGGGGGTTGTGGGGGG-5',
 5'-GGTGTGTGTTGGTGGGGTGGGGGTTGTGGGGGG-3'
 5'-GGGTGGGTGGTGTGGGGGGTTGTTTGGGGT-3',
 3'-GGGTGGGTGGTGTGGGGGGTTGTTTGGGGT-5',
 3'-TGGGGTTGGGTGGTTGGTTTGTGTTTTGGGGGGGGT-5',
20 5'-TGGGGTTGGGTGGTTGGTTTGTGTTTTGGGGGGGGT-3'
and fragments and analogues thereof.

 42. A method of suppression of expression of the
nerve growth factor receptor comprising the step of
25 administering the synthetic oligonucleotide of claim 3 in
sufficient amount for cellular uptake and binding to the
target sequence.

 43. The method of claim 42 for suppressing the
30 gene encoding nerve growth factor receptor, wherein the
synthetic oligonucleotide is selected from a group
consisting of:

 5'-GGGTTGTGGGTGGTGGGGGGGTTGGGTGTGTGG-3',
 3'-GGGTTGTGGGTGGTGGGGGGGTTGGGTGTGTGG-5',
35

1 5'-TGGGGGGGTTGGGTGTGTGGGTGTTTGGGTGTTGG-3',
3'-TGGGGGGGTTGGGTGTGTGGGTGTTTGGGTGTTGG-5',
5'-TTGGGTGTTGGGTGGGTGTTGGGGTGGGGTGGGGGTT-3',
3'-TTGGGTGTTGGGTGGGTGTTGGGGTGGGGTGGGGGTT-5',
5 5'-GGGTGGGTTTGGGTGTGGTTGGGTGGGG-3',
3'-GGGTGGGTTTGGGTGTGGTTGGGTGGGG-5',
5'-GGGGGTGGGGGGGGTGGGGGGGGGTGGGGGGGG-3',
3'-GGGGGTGGGGGGGGTGGGGGGGGGTGGGGGGGG-5'

and fragments and analogues thereof.

10

44. A method for arresting final replication of the Herpes Simplex Virus 1 comprising the step of administering the synthetic oligonucleotide of claim 3 in sufficient amount for cellular uptake and binding to the target sequence.

15

45. A method of claim 44 for arresting viral replication, wherein the synthetic oligonucleotide is selected from a group consisting of:

20

3'-TTTTTGTGTTGGGGGGTGGGGTGTGGGGGGTGT-5',
5'-TTTTTGTGTTGGGGGGTGGGGTGTGGGGGGTGT-3',
3'-TTTTTGTGTTGGGGGGTGGGGTGTGGGGGGTGT-5',
5'-TTTTTGTGTTGGGGGGTGGGGTGTGGGGGGTGT-3',
25 5'-TTTTTGGGGGGGGGGGGGGGGTGGGTGTTGGGTGGG-3',
3'-TTTTTGGGGGGGGGGGGGGGGTGGGTGTTGGGTGGG-5',
5'-TTTTTTTGTGTGTTGGGGTTGGGTGGGTGTTGTGGT-3',
3'-TTTTTTTGTGTGTTGGGGTTGGGTGGGTGTTGTGGT-5',
3'-TGGTGTGTTGTGGGTGGGTGGGTGTTGTGTTTTTT-5',
30 5'-TGGTGTGTTGTGGGTGGGTGGGTGTTGTGTTTTTT-3',
5'-TTGGGGGGGGGGGGGGGGGGTTTTGTGTTGTGTT-3',
3'-TTGGGGGGGGGGGGGGGGGGTTTTGTGTTGTGTT-5'

and fragments and analogues thereof.

35

1 46. The method of suppressing beta globin genes
synthesis in thalassemics and sickle cell anemia
comprising the step of administering the synthetic
oligonucleotide of claim 3 and sufficient amount for
5 cellular uptake combining to the target sequence.

47. The method of claim 46 for suppressing the
synthesis of beta globin gene, wherein the synthetic
oligonucleotide is selected from the group consisting of:

10

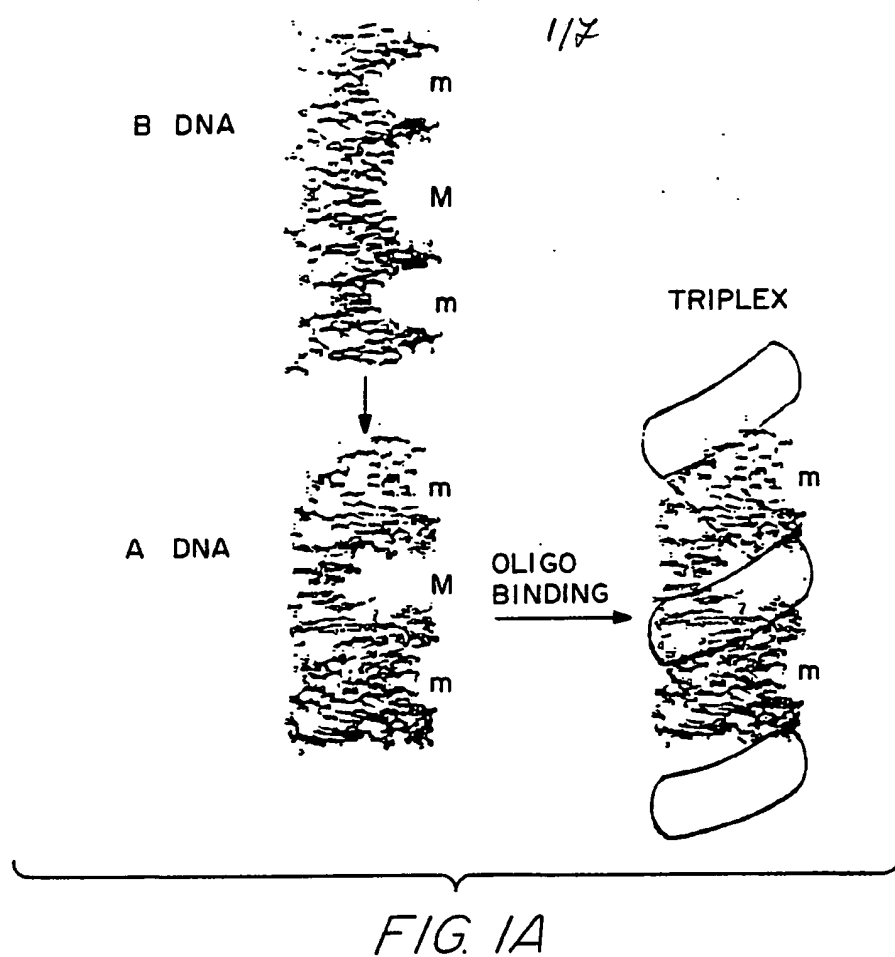
3'-GGTTTGGGGTGGTTGGGGTGTGTTGT-5',
5'-GGTTTGGGGTGGTTGGGGTGTGTTGT-3',
5'-TGGTGGTGGGTGGGGTGGTGGGTGGGGTGGGGTTTTTG-3',
3'-TGGTGGTGGGTGGGGTGGTGGGTGGGGTGGGGTTTTTG-5',
15 5'-TGGGGTGGGGTTTTTTGTGTGGGGTGTG-3',
3'-TGGGGTGGGGTTTTTTGTGTGGGGTGTG-5',
3'-GGGTTGTTGTTTTGTTTGGGGTTGTTTGT-5',
5'-GGGTTGTTGTTTTGTTTGGGGTTGTTTGT-3',
3'-TTGTTGGTTTGTTTTTTTTTTGTTGTGG-5',
20 5'-TTGTTGGTTTGTTTTTTTTTTGTTGTGG-3',
3'-GTGGGTGTTTTTTTTTGTTTTTTTTTT-5',
5'-GTGGGTGTTTTTTTTTGTTTTTTTTTT-3'

and fragments and analogues thereof.

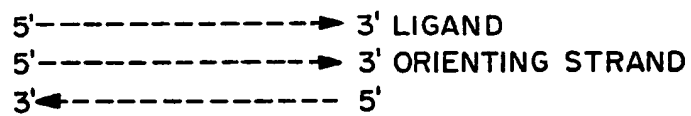
25

30

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PARALLEL



ANTIPARALLEL

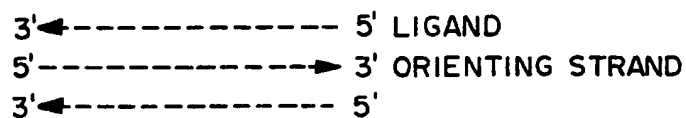


FIG. 1B

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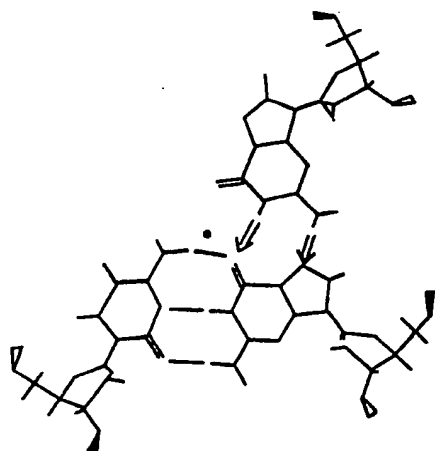


FIG. 2A

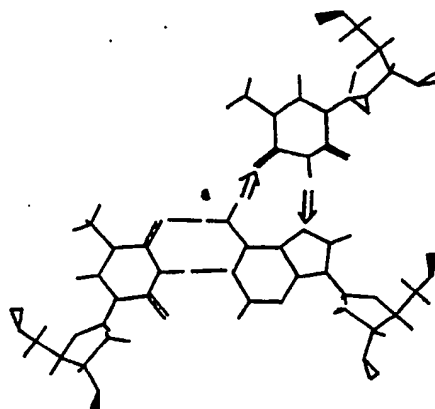


FIG. 2B

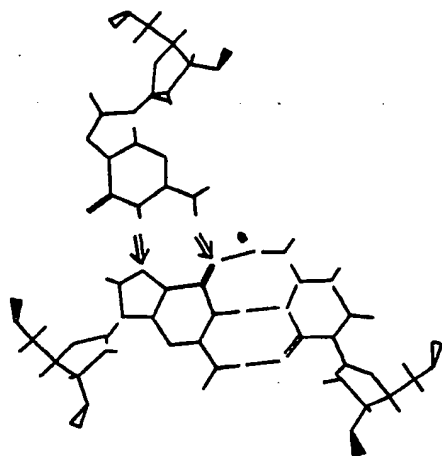


FIG. 2C

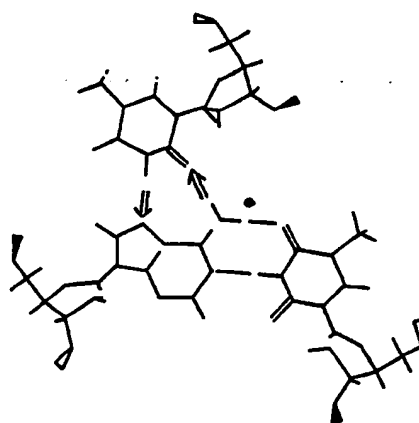


FIG. 2D

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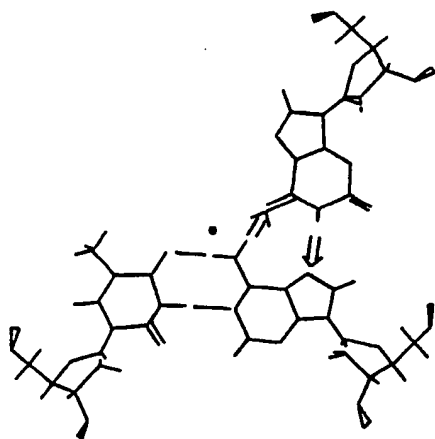


FIG. 3A

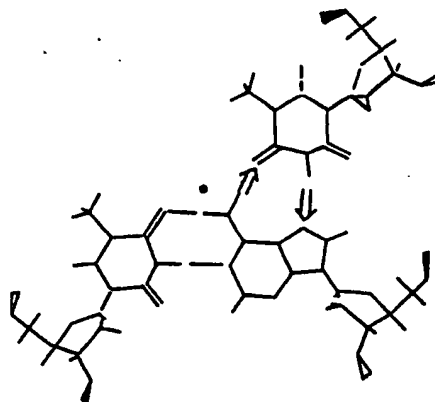


FIG. 3B

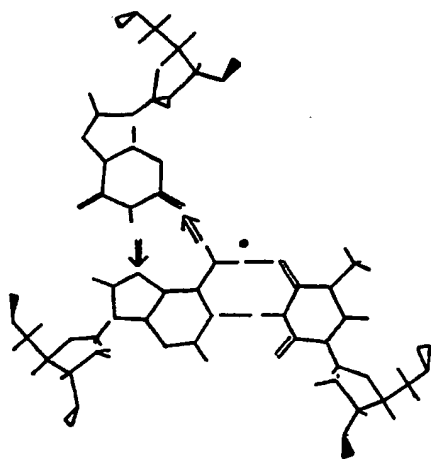


FIG. 3C

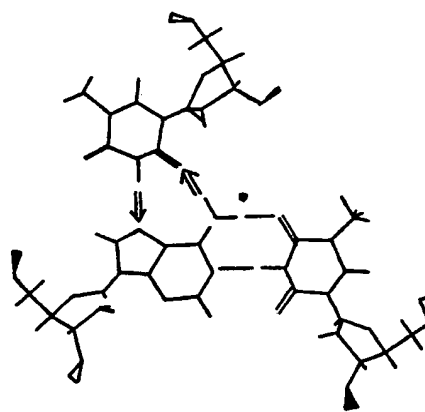


FIG. 3D

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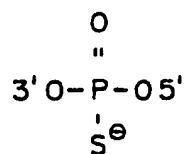


FIG. 4A

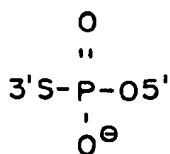


FIG. 4B

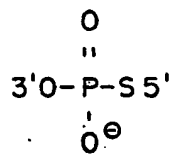


FIG. 4C

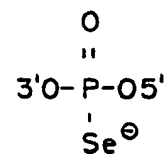


FIG. 4D

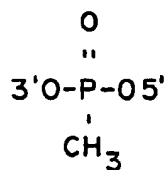


FIG. 4E

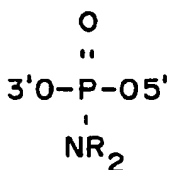


FIG. 4F

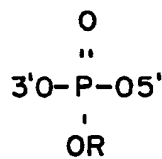


FIG. 4G

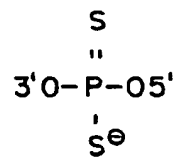


FIG. 4H

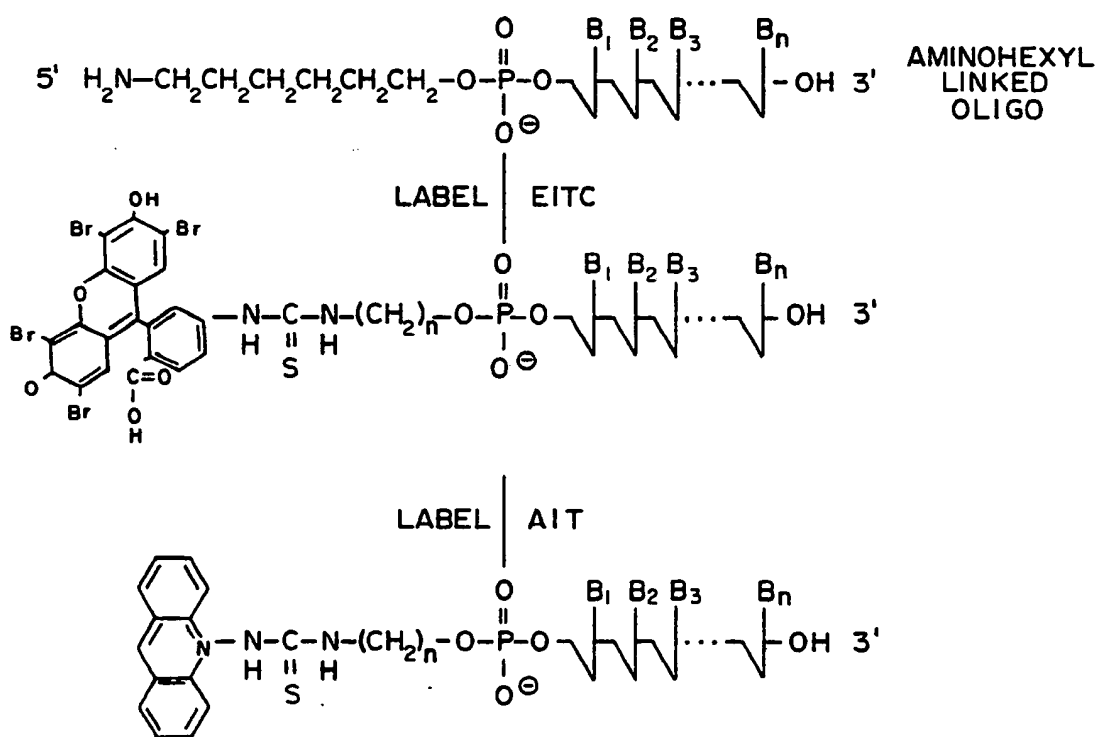


FIG. 5

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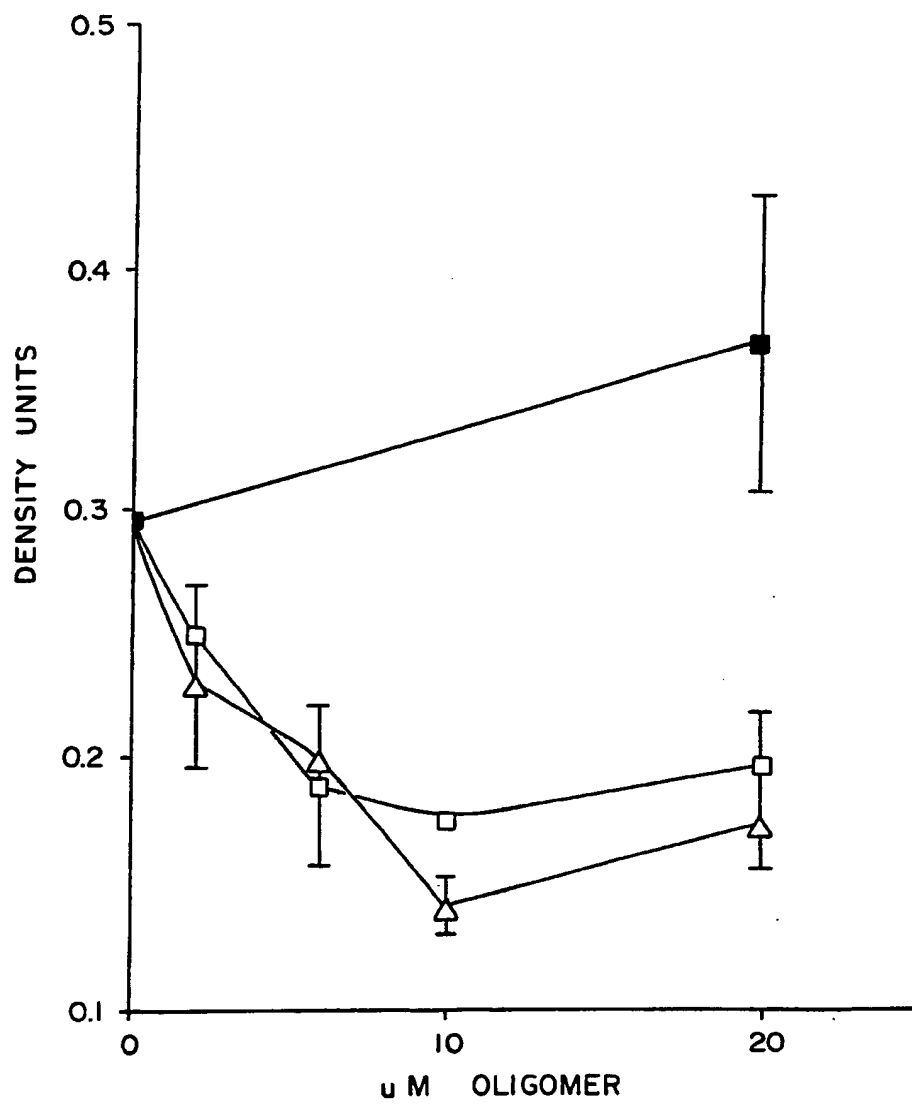


FIG. 6

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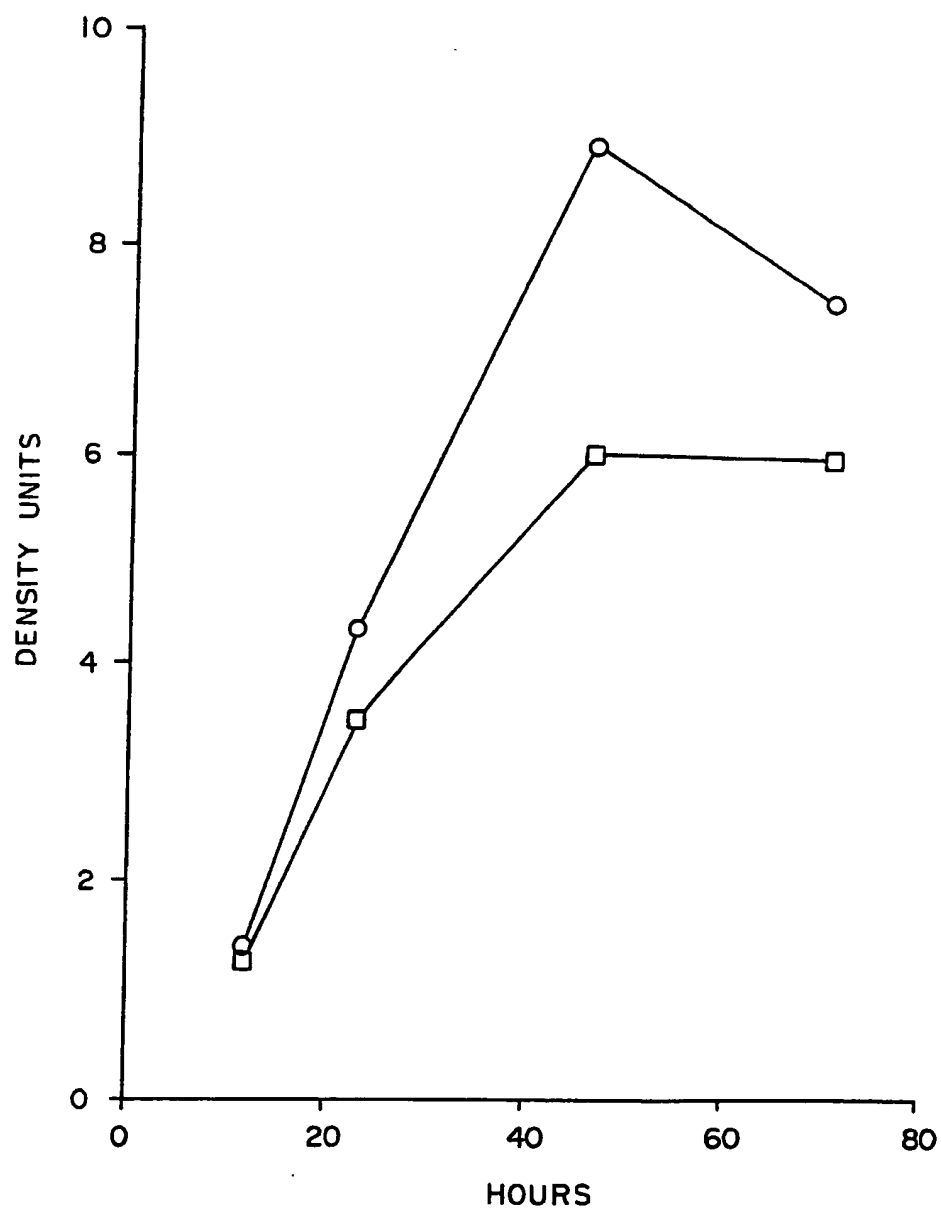


FIG. 7

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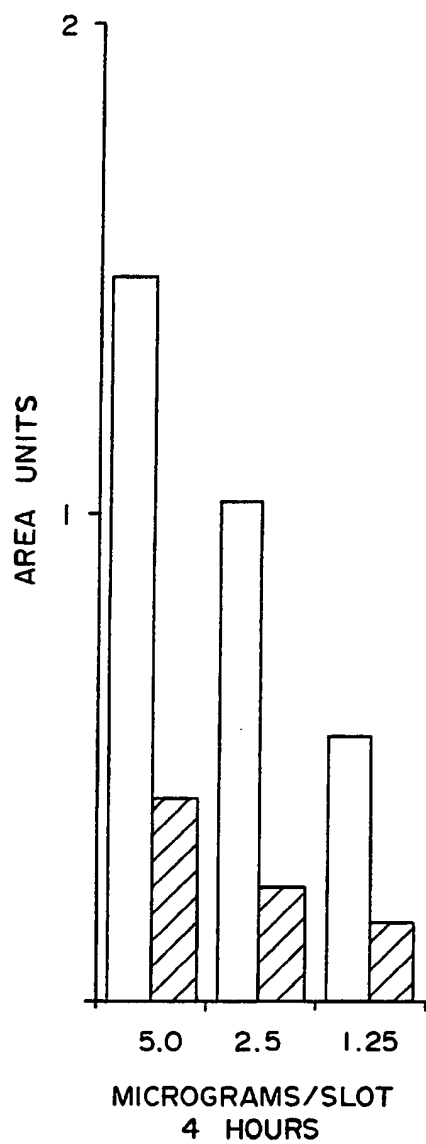


FIG. 8A

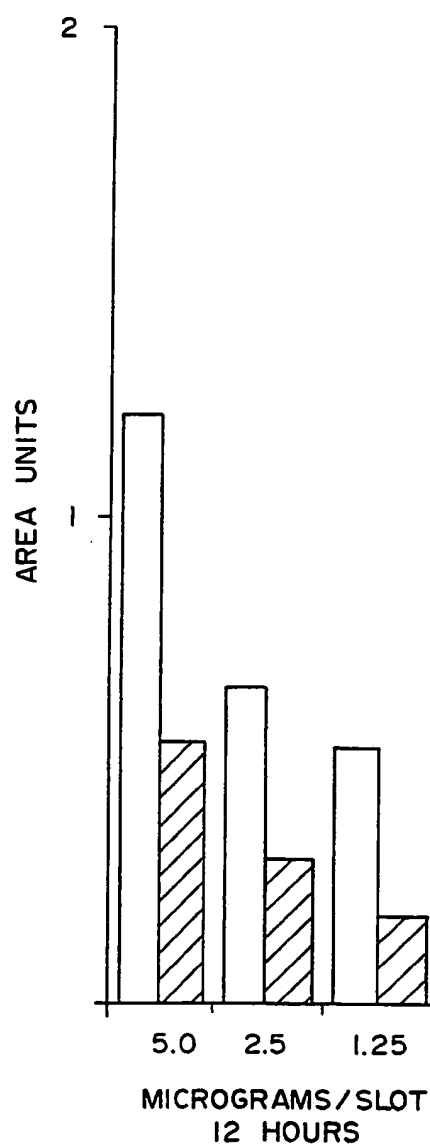


FIG. 8B

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/05769

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07 H 15/12; A22 C 9/00; C12N 15/00; A61 K 48/00

US: 536/27; 935/33; 424/405; 17/27; 514/44; 514/815; 514/950

II. FIELDS SEARCHED

Minimum Documentation Searched †	
Classification System	Classification Symbols
US	536/27, 935/33, 514/44, 424/405, 514/80 514/950, 17/27, 514/815

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

CAS, APS, GENBANK, EMBL

III. DOCUMENTS CONSIDERED TO BE RELEVANT †

Category *	Citation of Document, † with indication, where appropriate, of the relevant passages ‡	Relevant to Claim No. ‡
X Y	Journal of Experimental Pathology, volume 2 issued 1985; Minton "The Triple Helix: a Potential mechanism for Gene Regulation" see page 14	3 9
X Y	Physical Chemistry of Nucleic Acids, issued 1974 by Bloomfield et al. Harper & Row Publishers NY NY see pages 322, 323 and 331.	3,4 5,9
X	Nucleic Acids Research, volume 16, number 24, issued June 1988; Francois et al. "Sequence- specific recognition of the major groove of DNA by oligodeoxynucleotides via triple helix formation. Footprinting studies" pages 11431-11440 see abstract.	10, 14
X	Gene, volume 72, issued November 1988, Vlassov et al. "Sequence specific chemical modifications of double stranded DNA with alkylating oligodeoxy- nucleotides derivatives" pages 313-322 see abstract.	3,14

* Special categories of cited documents: †

"A" document defining the general state of the art which is not
considered to be of particular relevance

"E" earlier document but published on or after the international
filing date

"L" document which may throw doubts on priority claim(s) or
which is cited to establish the publication date of another
citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or
other means

"P" document published prior to the international filing date but
later than the priority date claimed

"T" later document published after the international filing date
or priority date and not in conflict with the application but
cited to understand the principle or theory underlying the
invention

"X" document of particular relevance: the claimed invention
cannot be considered novel or cannot be considered to
involve an inventive step

"Y" document of particular relevance: the claimed invention
cannot be considered to involve an inventive step when the
document is combined with one or more other such docu-
ments, such combination being obvious to a person skilled
in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

Date of Mailing of this International Search Report

25 MARCH 1989

22 MAY 1990

International Searching Authority

Signature of Authorized Officer

ISA/US

ROBERT A. WAX

III DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication where appropriate, of the relevant passages	Relevant to Claim No
X	Proceedings National Academy of Sciences, volume 85, issued March 1988, Praseuth et al. "Sequence-specific binding and crosslinking of and oligodeoxynucleotides to the major groove of DNA via triple helix formation" pages 1349-1353, see abstract.	3,9,14
X	Nucleic Acids Research, volume 16, number 8, issued May 1988 Stein et al. "Physicochemical Properties of Phosphorothionate Oligodeoxynucleotides" pages 3209-3221, see abstract.	3,8,9
X Y	Proceedings National Academy of Sciences, volume 85, issued 1987, Matsukura et al. "Phosphorathionate analogs of oligonucleotides: Inhibitors of replication and cytopathic effects human immunodeficiency virus" pages 7706-7710, see abstract.	3,8,9,21 22,44,45
X	Proceedings National Academy of Sciences, volume 85, issued July 1988. Walden et al. "Role of RNaseH in hybrid-arrested translation by antisense oligonucleotides" pages 5011-5015, see abstract.	3,46,47
X	Proceedings National Academy of Sciences, volume 85, issued February 1988. Wickstrom et al. "Human promyelocytic leukemia HL-60 cell proliferation and c-myc protein expression are inhibited by an antisense pentadecadeoxynucleotide targeted against cmyc mRNA" pages 1028-1032, see abstract.	3,17,18,19
X	Proceedings National Academy of Sciences, volume 81, issued 1984, Asseline et al. "Nucleic acid-binding molecules with high affinity and base sequence specificity: intercalating agents covalently linked to oligodeoxynucleotides" pages 3297-3301, see abstract.	3,13,14,16

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____ because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____ because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out^{1,2}, specifically:

3. ☐ Claim numbers _____ because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING³

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

1) Claims 1-20 entail a method of making an oligonucleotide, an oligonucleotide, and a method of treating cancer, classified in 514/80, 514/44, and 536/27 among others.

2) Claims 21-24: antibiotic treatment, classified in 536/27, 935/33, 514/44, and 424/405.

3) Claims 25-27: epidermal tissue treatment, classified in 514/44 and 514/950.

4) Claims 28-29: gene treatment, classified in 935/33 and 514/44.

5) Claims 30-31: meat treatment aids, classified in 17/25, 935/33 and 514/44.

6) Claims 32-33: interleukin receptor modification, classified in 935/33 and 514/44.

7) Claims 34-35: method of treating Alzheimers disease, classified in 935/33 and 514/44.

8) Claims 36-37: method of affecting the expression of epidermal growth factor, classified in 935/33 and 514/44.

9) Claims 38-39: method of repressing the function of GSTpi gene, classified in 935/33 and 514/44.

10) Claims 40-41: method of affecting cholesterol synthesis, classified in 935/33 and 514/44.

11) Claims 42-43: method of affecting nerve growth, classified in 935/33 and 514/44.

12) Claims 44-45: antiviral agents, classified in 935/33 and 514/44.

13) Claims 46-47: globin affecting agents, classified in 514/815, 935/33 and 514/44.

All of these inventions pertain to a different invention. Each of the different inventions forms a separate inventive identity and requires a different search of the patent classes as well as a separate search of the Genbank and EMBL databases.